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Supplement 255 — *Hovind-Hougen Kari.* Determination by Means of Electron Microscopy of Morphological Criteria of Value for Classification of Some Spirochetes, in Particular Treponemes. Pp. 41. 1976. (Section B)

Supplement 256 — *Danø Keld.* Experimentally Developed Cellular Resistance to Daunomycin. Resistance mechanisms, the daunomycin-pump and cross resistance to adriamycin, vincristine and vinblastine. Pp. 80. 1976. (Section A)

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CYTOPHOTOMETRIC STUDIES OF THE DNA, NUCLEIC ACID AND PROTEIN CONTENT OF LIVER CELL NUCLEI FROM PATIENTS WITH VIRUS HEPATITIS

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Ranek, L. Cytophotometric studies of the DNA, nucleic acid and protein content of liver cell nuclei from patients with virus hepatitis. Acta path. microbiol scand. Sect. A, 84 1–8, 1976.

With a view to investigating some of the causes why the size of liver cell nuclei increase in virus hepatitis, the nuclear size and the nuclear contents of DNA, nucleic acid and protein were measured by cytophotometry The liver cell nuclei could be grouped in classes according to their contents of DNA, nucleic acid, and protein and, as in control livers, diploid nuclei were always most frequent. Nuclei with intermediate DNA values, probably S-phase nuclei, were more frequent in hepatitis livers than in controls. The average size of nuclei from patients with hepatitis was significantly larger than that from controls, whereas the DNA content was the same. A significant, positive correlation between nucleic acid content, protein content and nuclear area was found. The high correlation between nuclear protein content and nuclear size simultaneously with the increased nuclear size during hepatitis is assumed to reflect an increased nuclear function.

Key words Hepatitis, virus liver cells cytophotometry

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In the first descriptions of the histological changes seen in liver biopsies from patients with acute virus hepatitis, a greater than normal variation in the size of liver cells and liver cell nuclei was mentioned (18) It has been shown (15) by karyometry that this pleomorphism to a large extent is due to a larger variation of hepatic nuclear size within each ploidy class and to an increased frequency of polyploid nuclei. To verify a variation within ploidy classes, DNA measurements are necessary Furthermore, it has been argued that the enlargement of the liver cell nuclei mainly is due to a higher nuclear DNA content (1) By microspectrophotometry it has been found that the DNA content in liver cell nuclei was higher than that in diploid mesenchymal liver cells (10 11) However a total shift to higher ploidy classes seems rather unlikely

It was the purpose of the present work to investigate the nuclear contents of DNA as well as the total amount of nucleic acid and protein in liver cells from patients with virus hepatitis, to see how the observed changes in nuclear size were correlated with the nuclear contents of the mentioned substances and to

TABLE 1 *Total Number of Liver Cell Nuclei Investigated and Average Coefficient of Variation of Diploid Nuclear DNA, Total Nucleic Acid and Protein Content and Diploid Nuclear Area in Nuclei Prepared by Different Methods*

	Nuclei prepared by method (No. of nuclei/ No. of slides)	Average coefficient of variation (range) diploid nuclei	
		nuclear content	nuclear area
DNA (Feulgen)	A (1539/30)	5.3 (2.4–13.0)	11.9 (5.7–17.9)
	B (691/15)	7.4 (3.3–13.0)	12.5 (8.3–16.0)
	C (471/11)	4.2 (2.6–6.1)	15.6 (8.5–25.8)
	D (148/4)	4.2 (3.2–4.7)	20.7 (17.3–26.2)
DNA (RNA-se, gallocyanin)	B (241/6)	5.7 (2.6–8.1)	13.5 (7.5–19.1)
	C (126/4)	4.4 (3.1–5.5)	15.9 (11.9–19.4)
Total nucleic acids (gallocyanin)	B (437/10)	7.3 (4.0–11.3)	12.4 (8.3–15.9)
Total protein (Naphtol-yellow)	B (297/7)	18.5 (9.5–26.8)	16.4 (13.8–19.4)

A = imprints, B = sucrose-Triton X 100, C = sodium tetraphenylboron, D = foetal liver cell homogenates.

compare the findings with those obtained in a control series (14).

MATERIAL

The material was derived from 45 liver biopsies from 37 patients with acute virus hepatitis. Two patients had a liver biopsy performed three times during a protracted course of the disease, two biopsies were obtained from four patients and one biopsy was obtained from the rest. Besides the histological diagnosis of hepatitis, the clinical symptoms and laboratory data also were consistent with virus hepatitis. In 12 of the patients, the hepatitis was supposed to be type B, being type A in the rest.

Judged on the basis of the histological criteria, hepatitis was of mild degree in three patients, while it was of moderate degree in 19 and severe in 15. Clinically, two patients were in hepatic coma; both survived and recovery was complete. The disease ran a protracted course in three patients, but recovery was complete in all cases. The remaining patients had a clinically mild hepatitis, and chronic liver disease did not develope in any of these. Drug induced hepatitis was not suspected in any patient included in the present series. The mean age of the patients was 34 years (range 14–79). 19 were females and 18 males. Human, foetal liver cells were investigated with a view to assessing the frequency of S-phase nuclei in rapidly proliferating liver tissue.

A total of 3984 nuclei in 87 slides was measured (Table 1). The control material which consists of liver biopsies from patients without liver disease has been described previously (14).

METHODS

Liver biopsies were obtained from among the routine liver biopsies performed in the department.

The biopsy was obtained from the fasting patient between 8 and 9 a.m., using a 1.6 mm Menghini needle. The tissue was immediately divided into two parts, one, weighing between five and ten mg, was used for the present investigation, the other being used for routine histological examination.

Details of the procedures for the preparation of liver cells or liver cell nuclei has been described previously (14).

Whole liver cells were prepared either as imprints (method A in Table 1) or by homogeniza-

tion in sodium-tetraphenylboron (method C in Table 1)

Isolated liver cell nuclei were prepared by homogenization in sucrose followed by treatment with Triton X 100 (method B in Table 1) The liver cells or liver cell nuclei from the homogenates were placed on glass slides by centrifugation in a Shandon cytocentrifuge.

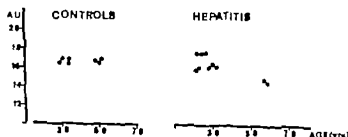
Homogenates from four human foetal livers (spontaneous abortions the gestation age of which ranged between three and four methods) were obtained from others (6) and used directly for cytocentrifugation.

All slides were fixed in Carnoy's solution for 15 minutes DNA was determined after Feulgen staining (14) or on RNA-se treated, gallocyanin stained nuclei (4 9) Total nucleic acid was determined on gallocyanin stained (4) nuclei or on unstained nuclei by ultraviolet microspectrophotometry (19) Total protein was determined after naphtol-yellow staining (2)

Microspectrophotometry was performed in a Zeiss UMSP I at maximum absorption wave length. The nuclei were scanned in fields of one micron, with one micron between the scanning lines. The extinction values obtained from the scan points were automatically integrated and the area of the nuclei was considered the total frequency of scan fields with an extinction value abov a present threshold, giving the approximate area in μm^2 Means and standard deviations of nuclear contents in arbitrary units (AU) and area (μm^2) of each ploidy class with more than five nuclei were calculated The variation in nuclear contents is expressed as coefficients of variation $(SD/\bar{x})100$. Correlation between nuclear content and nuclear area was estimated by a non-parametric test, the Spearman rank-correlation coefficient with correction for ties (20)

RESULTS

Nuclear DNA content In all slides investigated, the liver cell nuclei were distinctly separated in ploidy classes. The average frequency of di tetra and octaploid nuclei was 89 7.8 and 0 8 per cent, respectively Diploid nuclei were always the dominant ploidy class. The Feulgen-DNA value of lympho- and leucocytes found among the liver cell nuclei was used to establish the diploid value Nuclei with a DNA value 2.5 times the standard deviation above the mean diploid value or 2.5 times the standard



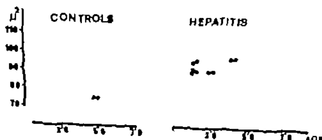


Fig 1 Average Feulgen DNA values in arbitrary units (AU) and average size (μm^2) of diploid nuclei from imprints of liver biopsies in 12 control patients and 24 patients with acute virus hepatitis. The age of the patients appears from the horizontal axis.

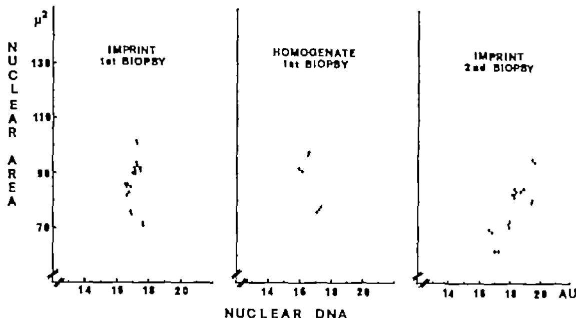


Fig 2 Feulgen DNA content versus nuclear area of diploid nuclei. To the left, the results from an imprint of the liver biopsy taken during the acute phase of virus hepatitis. In the middle results of measurements of isolated liver cell nuclei from the same biopsy. To the right, measurements obtained from an imprint of another liver biopsy taken from the same patient in the recovery phase of the disease. The nuclear area has decreased significantly

deviation above or below the mean tetra and octaploid value were defined as S-phase nuclei. In the whole material of liver biopsies, an average of two per cent S-phase nuclei was found. In foetal livers S-phase nuclei comprised 17 per cent. Nuclei with a DNA content 2.5 times the standard deviation below the mean diploid DNA value were defined as hypodiploid and comprised 0.3 per cent. Besides the types of nuclei mentioned, *two mitoses presenting tetraploid* DNA values and two presenting 16-ploid nuclei were found.

In Fig 1 (top) the average DNA values of *diploid* liver cell nuclei from 24 imprints are shown together with the results obtained in a control series. The average DNA value of diploid nuclei was not different from that of the controls (16.1 and 16.3 arbitrary units respectively)

The average coefficient of variation of the DNA content (Table 1) varied from 4.2 to 7.4 per cent, dependent on the isolation procedure, being approximately the same for di- and tetraploid nuclei DNA determinations at RNA-se treated gallocyanin stained nuclei did not differ from the Feulgen stainings as regards variation in DNA content.

Nuclear total nucleic acid content By UV spectrophotometry of 34 nuclei from two patients, an average nuclear content of nucleic acids of 8 pg was found. This is insignificantly higher than that found in control livers (7 pg) and still consistent with a diploid DNA value.

In the gallocyanin stained slides, the ploidy classes were clearly separated, as seen also in the Feulgen stainings, and the ratio between diploid- and tetraploid values was close to 1 2.

Nuclear protein content Whereas the values of the DNA and total nucleic acids were grouped rather closely around mean ploidy levels, the values denoting the protein content of the nuclei scattered widely and separation into ploidy classes was made after visual inspection of the plots. The average coefficient of variation of the protein content

CORRELATION BETWEEN NUCLEAR CONTENT AND NUCLEAR AREA

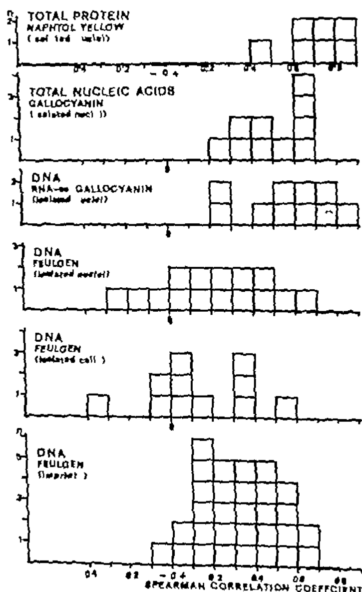


Fig. 3 Histogram of the Spearman rank-correlation coefficient between nuclear contents of DNA, nucleic acid and protein and area of diploid nuclei, evaluated by different isolation and staining procedures (see text). White columns indicate insignificant correlations, dark columns indicating significant positive correlations.

was 18.5 per cent. It was not possible to point out S-phase nuclei in protein stained nuclei.

Nuclear size. The average size of diploid nuclei from Feulgen imprints (first biopsies only) are shown in Fig. 1 (bottom) together with the results obtained in the control series. The average nuclear area was significantly ($p<0.001$) greater in the present material (96 and 79 μm^2 respectively). The nuclear size was not correlated with the age of the patient.

The average coefficient of variation of nuclear area was approximately 12–16 per cent,

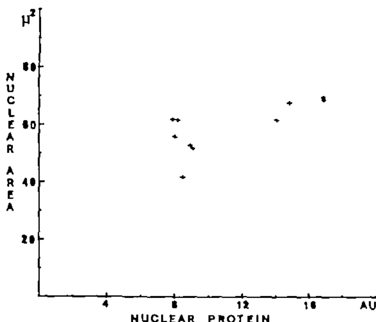


Fig 4 Nuclear protein content in arbitrary units (AU) versus nuclear area (μm²) of diploid nuclei measured on liver cell nuclei isolated from a patient with acute virus hepatitis.

being of the same magnitude in di and tetraploid nuclei The nuclear size overlapped between ploidy classes.

Correlation between nuclear contents and nuclear size In Fig 2, the individual values of the Feulgen-DNA content and nuclear size of diploid nuclei from three samples of nuclei from the same patient are plotted. The nuclear area is significantly greater ($p<0.001$) in the first biopsy than in the second. The Spearman rank-correlation coefficient between nuclear DNA content and nuclear area is +0.035 −0.164 and +0.482 respectively (from left to right) In Fig 3 the Spearman rank-correlation coefficients between the nuclear contents and nuclear area of diploid nuclei are shown as histograms.

The correlation between DNA content and nuclear area was significantly positive in 34 out of 66 slides. The highest frequency of significantly positive correlations between DNA content and area was found in RNA-se treated, gallocyanin stained nuclei while the lowest frequency was found in Feulgen stained isolated cells In the whole material, the correlations between nuclear protein content and nuclear area were found to be highest and, in all seven slides where nuclear protein was investigated, a correlation between the protein content of diploid nuclei and diploid nuclear area was found to be significantly positive.

In Fig 4 the results from one of the biopsies is shown (diploid nuclei only) The Spearman rank-correlation coefficient between nuclear protein content and nuclear size is +0.721 in this case

DISCUSSION

The present investigation confirms that the liver cell nuclei are enlarged during acute virus hepatitis, as found by karyometry (12, 15) This enlargement is not due to an increased DNA content as claimed by some investigators (1) or to a shift of the prevailing ploidy class from diploid to tetraploid as claimed by others (10, 11) The relatively high correlation between nuclear protein content and nuclear size indicates that the enlargement of the nuclei is due mainly to a higher protein content. By interferometry I have shown (16) that dry weights of these liver cell nuclei are significantly higher than those of control nuclei, which is in support of this concept. It may be assumed that the in-

crease in nuclear size and the high correlation with the nuclear total nucleic acid and protein content is due to an increased nuclear function. This interpretation is supported by findings in animal experiments (21) where a highly significant correlation between H^3 phenylalanin incorporation and nuclear size has been demonstrated, and by studies of the amino acid incorporation in liver biopsies from patients with virus hepatitis in whom the protein synthesis rate was found (17) to be increased.

It appears from Fig 1 that there is no tendency towards a decrease in nuclear size with age, as otherwise reported by *Denkhaus* (3) In *Loetschke's* karymetric study (12) of human liver cell nuclei from 17 patients, such decrease in nuclear size was not found, thus supporting the findings obtained in the present study It also appears from Fig 1 that the enlargement of the diploid nuclei during virus hepatitis was found in all age groups.

By Acridin-orange fluorescence cytophotometry of human liver cell nuclei *Heimer* (7) found increasing repression of the genetic material with age According to findings in the present study such repression does not seem to affect the ability of the liver cell nuclei to increase in size probably owing to an increased metabolic activity as mentioned previously

Thymidine incorporation was not studied in this investigation, but it applies to approximately two per cent of the nuclei that the DNA values indicated that they were S-phase nuclei. The frequency of S-phase nuclei in livers affected by hepatitis was significantly higher than that in control livers (0 6 per cent) but much lower than that found in proliferating liver tissue from foetal livers where 17 per cent S-phase nuclei were found. The existence of nuclei with hypodiploid values was not demonstrated in the control material (14) these nuclei, which most often were found in biopsies presenting many liver cell necroses, represented probably necrotic liver cell nuclei, as demonstrated in animal experiments after ischaemic necrosis of the liver (8)

In this as well as in a previous work (14) the methods by which to obtain nuclei for cytophotometry have been different. As regards Feulgen DNA determinations, imprints, isolated liver cells and isolated nuclei may be used. The isolation procedure influences to some degree the staining intensity and, as pointed out in a previous paper (14) and in works by others (5 13) the Feulgen stainings may vary significantly from one slide to another although the staining procedures were rigorously standardized. As regards the Feulgen-DNA content of di- and tetraploid nuclei, however a ratio close to 1 2 was always found and used as a control of the stochiometry of the staining in each slide.

The nuclei isolated by means of Triton X 100 are especially suitable for the determination of nucleic acids and protein as the high concentration of RNA and protein in the cytoplasma of liver cells makes cytophotometric determination of these substances in the nuclei from whole cells difficult.

In conclusion, the cytophotometric investigations have shown enlargement of the liver cell nuclei and a significantly positive correlation with the nuclear protein content and a higher frequency of nuclei presenting intermediate DNA values. These changes are assumed to represent an increased metabolic and regeneratory activity respectively

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I am indepted to Mrs. *H Wildschiødtz* for expert technical assistance.

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HETEROTRANSPLANTATION OF HUMAN MALIGNANT MELANOMAS TO THE MOUSE MUTANT NUDE

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Povlsen, C. O Heterotransplantation of human malignant melanomas to the mouse mutant nude. Acta path. microbiol. scand. Sect. A, 84 9–16, 1976.

In 32 experiments involving transplantation of human malignant melanomas to the mouse mutant nude, tumourtake was observed in 14 instances. The tumour tissue which took and grew after inoculation was derived from malignant melanoma metastases of the skin (10 takes from 13 transplantations) and from primary malignant melanomas of the eye (4 takes from 6 transplantations) Not one of 13 attempts to transplant primary tumours of the skin was successful. In 7 instances serial transfers of transplanted tumour were successful, the degree of success ranging from 3 to 41 passages. Some passages were wilfully interrupted but two are continuing. The transplanted tumours grow locally Neither lymph node metastases nor organ metastases have been observed. Histological appearances of the transplanted tumours, also after serial passages, were identical with those of the original human donor material. The human tumour/nude mouse system is contrasted and compared with other heterotransplantation models.

Key words Heterotransplanted human malignant melanomas nude mice.

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The mouse mutant nude which displays recessive thymus aplasia (4, 11) has been shown to accept transplants of human malignant tumours (5–7 12–15 17)

There are, as yet, but few systematic studies of the transplantation of human tumours to the nude mouse. Such studies are necessary to evaluate the place of this new model in cancer research. Of particular interest and importance are studies involving cancer chemotherapeutic agents, but it is first necessary to catalogue those human tumours where take is reliable and reproducible after transplantation to the nude mouse

Earlier studies have concerned transplantation of adenocarcinoma of the colon, the rectum and epidermoid carcinomas (12, 13)

The following records experience derived from experiments in which malignant melanoma tissue from 32 patients was transplanted to the nude mouse.

MATERIAL AND METHODS

Mice Recipient animals were 5–10 weeks old BALB/c nude mice of both sexes bred at the Pathological Anatomical Institute, Kommunehospitalet, Copenhagen. These animals were the outcome of the 1st to 7th backcross cycle in a gene transfer to BALB/c mice (16) The mice were

TABLE 1 *Results of Transplantation of Human Malignant Melanomas to Nude Mice*

Case No.*	Patient Data	Site of primary tumour	Tissue used for transplantation	Fate of graft	Transplant generations
1	36 ♂ P 3515	Skin	Primary tumour	–	–
3	42 ♀ P 3517	–	–	–	–
4	59 ♂ P 3518	–	–	–	–
5	72 ♀ KH 8508	–	–	–	–
6	27 ♀ P 3523	–	–	–	–
7	50 ♀ P 3531	–	–	–	–
8	51 ♂ P 3535	–	–	–	–
11	32 ♂ P 3645	–	–	–	–
16	69 ♂ P 3663	–	–	–	–
18	49 ♀ P 3652	–	–	–	–
21	60 ♂ P 3654	–	–	–	–
23	61 ♂ P 3655	–	–	–	–
24	7[illegible] ♂ P 3656	–	–	–	–
9	45 ♀ P 2938	–	Lymph node metastasis	+	6
10	30 ♀ P 2652	–	–	+	11
13	62 ♀ P 2937	–	–	–	–
14	30 ♀ P 3615	–	–	+	3
20	47 ♀ P 2596	–	–	+	1
22	61 ♂ P 3633	–	–	+	1
27	36 ♀ KH 6160	–	–	+	3
17	67 ♀ P 2789	–	Cutaneous metastasis	–	
2	48 ♀ P 2736	–	Subcutaneous metastasis	+	37
12	60 ♀ P 2800	–	–	+	41§
15	60 ♀ P 2824	–	–	+	1
19	45 ♀ P 292	–	–	–	–
32	69 ♀ P 341	–	–	+	4§
25	37 ♂ KH 639	Eye	Primary tumour	+	1
26	51 ♀ KH 916	–	–	+	1
28	55 ♂ KH 826	–	–	+	1
29	65 ♂ KH 857	–	–	+	1
30	74 ♂ KH 488	–	–	–	
31	77 ♀ KH 682	–	–	–	
				Total 14/32	

* Case no. refers to order in which transplantations were performed.
§ Tumours still being serially transplanted.

kept under conventional conditions as described earlier (16). Observations were made daily and post mortem studies were made in every animal.

Tumour tissue. The mice were inoculated with tumour tissue from 32 patients with malignant melanoma. These patients represent a consecutive available series treated in departments of plastic surgery, general surgical departments, and eye departments in hospitals from the Copenhagen area. The tumour inoculations were made over the period from 28.11.69 to 3.12.74. Various histological types of primary and secondary malignant melanoma were represented, and these can be described in 3 main groups (Table 1).

1) 13 primary malignant melanomas of the skin.

2) 13 metastases from primary malignant melanomas of the skin.

3) 6 ocular malignant melanomas of the choroid.

Inoculation method. Tumour tissue was inoculated into the nude recipient between 20 and 90 minutes after removal from the patient. Selection of the tumour tissue segment which appeared to be most suitable for transplantation was made macroscopically.

From primary malignant melanomas of the skin, strips were taken, 2 mm in breadth, to include both central and peripheral tumour tissue. When tumours were more than 3-4 mm in depth, tissue was taken primarily from the deeper aspect of the tumour, i.e. from tumour infiltrated corium and

ubcutis. Areas with ulceration and scab formation were recorded.

The criterion for selection of tissue from metastatic tumours, and from primary malignant tumours of the eye was macroscopic vitality

In the first 7 experiments mice were inoculated with 0.5 ml of a suspension of mechanically fine dealt tumour material in tissue culture medium (Glaxo 199) In all subsequent experiments solid tumour blocks, 1 × 2 × 2 mm, were transplanted as previously described (12) All inocula were placed directly subcutaneously in the flank with no proceeding period of culture.

The primary inoculations were made in 2–8 nude mice, and in 1 normal littermate. In the serial transplantation studies 2–20 nude mice and 1 normal littermate were inoculated, whenever sufficient tumour tissue was available. Inoculation of normal littermate was undertaken to confirm that the primary human tumour tissue was antigenic, and to allow a more complete cover study of possible murine transformation in serially transplanted tumour tissue.

The first 25 transplantations were made with animals under ether anaesthesia, and the last 7 under propanidid anaesthesia (Epontol® 0.5 mg/g body weight intraperitoneally)

At the time of primary transplantation to the nude mice histological preparation was made of adjacent tumour tissue from the patient to serve as a standard for comparison with subsequent histological preparations taken from tumours hosted by the nude mice Histological preparations were also made from organs which evidenced macroscopic change, and from enlarged regional lymph nodes. The tissues were fixed in formalin, embedded in paraffin wax, and 7 µm sections were cut and stained with hematoxylin and eosin and van Gieson-Hansen stain. Tumour tissue preparations were also stained for iron and with Lillie stain for melanin (9)

RESULTS

Fate of Tumourgrafts in Nude Mice

Table 1 records that the tumour tissue transplants were accepted by the nude mice in 14 of the 32 experiments.

Inocula of tumour cell suspensions (6 attempts) and solid tumour blocks (7 attempts) derived from primary malignant melanoma of the skin did not take.

Inocula of metastases from primary malignant melanomas of the skin were accepted and grew in 10 of 13 experiments. Take was observed both after injection of tumour cell suspension (1 attempt) and after inoculation of solid tumour blocks (9 attempts) In 7 cases (No 2, 9 10 12 14 27 and 32) serial transplantation of tumour tissue was possible. The number of transplant generations is shown in Table 1 Two serially grown tumours (No. 12 and 32) are being continued and further study is being made. The remaining serially grown tumours failed at some stage or were wilfully discontinued.

4 of 6 tumour transplants deriving from the choroid were accepted and grew but serial transplantation was unsuccessful.

Fate of Tumour Grafts in Normal Littermates

Neither primary tumour tissue transplants, nor transplants of tumour tissue after serial passage were accepted

Growth Patterns and Macroscopic Appearances of Tumour in Nude Mice

In those instances when inocula of metastases from malignant melanomas of the skin took, growth patterns were the same, both of the primary transplant and of the serially transplanted tumour Take of solid tumour blocks was apparent after 10–14 days, but 5–6 weeks elapsed before take of tumour cell suspensions could be confirmed. After both inoculation methods, freely movable swellings, clearly defined were seen at the inoculation site. These swellings increased evenly in size up to the time of the death of the animals. These growing tumours often attained considerable size. The largest tumour in the series measured 25 × 30 × 30 mm and weighed 17.5 g at the death of the animal. The rate of growth of individual tumours has been constant in the performed passages.

Inocula from primary melanomas of the choroid grew very slowly and at most came to measure 2–5 mm in diameter at the death of the animals (Fig. 1)

All tumours appeared to be encapsulated in the subcutaneous space, and could easily be enucleated. On cross section some tumours appeared light grey in colour with no pigmentation, while other displayed brown-black



Fig 1 Low power view of human malignant melanoma of the eye 43 days after transplantation to a nude mouse (case No 26, 1st transplant generation) The tumour is well circumscribed in the subcutaneous space 56 × H & E.

pigmentation. When tumours measured more than 5 mm in diameter central necrosis was a common finding Tumour growth was always local, and metastases to lymph nodes or organs have never been observed.

The larger tumours were an inconvenience, but otherwise without effect on the animal's general condition Survival time under the described conditions was between 4 and 6 months, i.e. 2–4 months after tumour inoculation. The animals in which tumours took, died with symptoms of the wasting syndrome, just as do their untreated nude sibs.

Microscopic Appearances of Tumours in Nude Mice

Histological studies showed that tumours lay in the subcutaneous space deep to the panniculus musculosus, resting on the abdominal musculature (Fig. 1)

The tumour tissue was solid, often displaying an alveolar cellular arrangement. Extensive central necroses of the larger tumours were often seen and these areas were richly infiltrated with polymorphnuclear granulocytes. All the tumours which took had sparse stroma, and only isolated mononuclear cells were seen.

One tumour (No. 12) displayed local infiltration of the striated musculature and fatty tissue. All other tumours, irrespective of size were fully defined, surrounded by a condensed layer of connective tissue No infiltration of blood or lymph vessels could be demonstrated.

Histological appearances of the original donor tumour tissue, and of the same tumour after primary and serial transplantation were identical within the limits of the study Melanin content was also as in the original donor tumour No differences could be pointed even in the tumour tissue which had undergone many serial passages (Figs. 2–3)

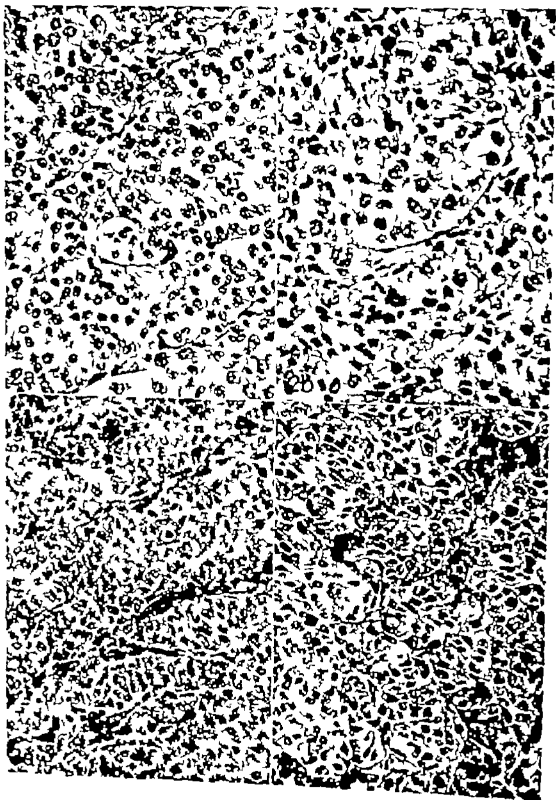
When tumour transplants did not take the inocula shrank, and could disappear completely In some instances small often darkly pigmented swellings persisted. Histological study of these tumour remnants showed macrophage infiltrated necrotic tissue often rich in melanin, but a total absence of tumour cells.

DISCUSSION

It is found that metastases from malignant melanomas of the skin can be transplanted to the nude mouse with a high degree of success

Fig 2 a & b Section from original tumour donor material of case No. 2 (a) and mouse grown tumour after 37 transplant generation in nude recipients (b) The microscopic pictures are very much alike 350 × H & E.

Fig 3 a & b Sections from operative specimen of case No. 32 ( ) and 4th transplant generation in nude mice (b) The histological appearance and the melanin content of the mouse passaged tumour is in accordance with the human donor material 350 × H & E.


2a
2b

(10 takes in 13 transplantation studies) 7 of these tumours survived 3 or more serial passages in the nude mouse, and two such tumours continue now after 41 passages to date

By contrast none of 13 transplantations of primary malignant melanomas of the skin was successful.

4 of 6 transplantations of tissue from primary malignant melanomas of the eye were successful, but none survived serial transplantation.

A possible explanation of these conflicting results is difference in stroma content of the tumours. All metastatic tumours were very poor in stroma, but the primary tumours of the skin consisted of islands of tumour cells, surrounded by a rich connective tissue stroma. Thus tumour diffusion nutrition immediately after transplantation might have been affected. This contention is to some extent supported by the success in transplantation of primary malignant melanomas of the eye—these tumours having very scant stroma.

This marked difference between take of primary and metastatic malignant melanomas of the skin conflicts with the findings of other authors. Thus *Sordat et al.* (17) made melanoma transplantations to BALB/c nude mice and report successful take of 7 of 8 metastatic tumours, and 4 of 6 primary melanomas of the skin. Two of these later tumours were successfully serially transplanted (*Sordat B.* personal communication 1974)

The rate of growth of transplanted tumours also varied extremely Metastatic skin melanoma tissue grew rapidly whereas that from primary tumours of the eye grew extremely slowly These growth patterns are in accordance with clinical experience of the respective tumours.

Histology of the transplanted tumours was identical with that of the original human tumour even after many passages. The majority of growing tumours remained defined without tendency to local infiltration. In one instance, however (case No 12) local invasion of striated muscle and fatty tissue was seen. Distant metastases to lymph nodes or organs were never observed. This is as found by *Sordat et al.* (17) and also in previous experiments with the nude mouse involving transplantation of adenocarcinoma from the colon and rectum, epidermoid carcinoma, and Burkitt's lymphoma tissue (12-15)

Giovanella et al. (5) found differently They injected nude mice with cell cultures derived from human malignant melanomas, and observed local spread of the tumours, which metastasized to the regional lymph nodes, and in one instance lung metastases were seen. *Giovanella et al* (7) also reported metastatic spread to lymph nodes of human malignant tumours directly transplanted to nude mice. In possible explanation, these authors propose that there could be differences in the general condition of animals and in survival time The back ground strain of the nude mice might also be significant. *Giovanella et al.* (7) used outbred C57BL/6 and Swiss nudes. In the present study and in *Sordat et al's* (17) study nude mice in a gene transfer to BALB/c mice were used.

Human malignant melanoma has been studied in other heterotransplantation systems, for example the anterior chamber of the eye in guinea pigs. Seven of 10 transplants of metastatic melanoma "took" but three transplants of primary malignant melanoma were unsuccessful (*Greene* (8))

Bradford Patterson (2) reported that 6 of 14 human malignant melanomas could be serially transplanted in the cheek pouch of hamsters. In all but one animal, cortison preparation was a pre requisite of tumour take *Williams et al.* (18) reports similarly that 4 of 8 malignant melanomas grew in the cheek pouch of hamsters, and also that the results were best in cortison prepared animals. No attempt was made to serially transplant tumours.

Berenbaum et al. (1) transplanted a number of different human tumours in thymectomized irradiated and antilymphocyte serum treated mice 13 of 16 malignant melanoma transplants grew but no attempts were made to transplant tumours serially

After culturing of cells from human malignant melanomas, *Mukherji* (10) reports 2

successful transplantation experiments in neonatal, antithymocyte serum treated rats. Serial transplantations were thereafter successful in up to 12 passages in similarly prepared neonatal rat recipients.

Cobb (13) transplanted different human malignant tumours in the subcutaneous room in thymectomized antithymocyte serum treated hamsters. 3 of 6 transplants of primary malignant melanomas of the skin were accepted, but no report is made of any attempt at serial transplantation.

Thus, it is apparent that similar degrees of success have been attained in other transplantation model systems as in the nude mouse system. The latter animal, however differs from all other in that no form of preparation is necessary. Operative preparation, cortison treatment and other form of immunosuppression, constitute an extra group of variables which could be thought to complicate interpretation of results. Furthermore, a standard experimental model is desirable so that meaningful comparison can be made between the findings of different research workers. The nude mouse is such a new laboratory animal that the majority of studies hitherto can only be described a pilot studies, but already there are pointers that the animal may prove to be an exceptionally valuable research animal.

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After culturing of cells from human malignant melanomas, *Mukherji* (10) reports ?

Acta path. microbiol. scand. Sect. A, 84: 17–27, 1976

LIGHT MICROSCOPIC AND ULTRASTRUCTURAL OBSERVATIONS ON THE SHORT-TERM EFFECTS OF ETHYLENE-1 HYDROXY-1, 1 DIPHOSPHONATE (EHDP) ON RAT TIBIA EPIPHYSIS

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Larsson, A. & Larsson, S. E. Light microscopic and ultrastructural observations on the short term effects of ethylene-1-hydroxy-1, 1-diphosphonate (EHDP) on rat tibia epiphysis. Acta path. microbiol. scand. Sect. A, 84: 17–27, 1976.

Ethylene-1-hydroxy-1, 1-diphosphonate (EHDP) was administered intraperitoneally to one-day old rats at a concentration of 30 and 40 mg/kg body weight/injection. Two groups of animals received four doses, respectively, over a 44-hour-period and a third group received two high doses with 15-hour interval, the total amounts corresponding to 30, 40 and 20 mg/P/kg body weight. Animals in the first two experimental groups showed increased width of the hypertrophic zone as compared with normal controls and lack of calcified septa within the zone of provisional calcification. In the calcification zone, persistent atypical chondrocytes were present and in the septa a large number of matrix vesicles which hardly ever contained any crystals. The ground substance of these septa contained fine precipitates, probably representing proteoglycan complexes which were not seen normally in this zone. In the third group, the septa of the calcification zone were calcified and the hypertrophic zone was not clearly widened. In the metaphysis, remnants of cartilage or osteoid were observed as "islands" covered with osteoblast-like cells which showed signs both of collagen and apatite formation, the latter evidenced by the appearance of crystals within the matrix vesicles. It is suggested that the complex effects observed in calcifying cartilage after administration of high doses of EHDP *in vivo* are not restricted solely to the stage of crystal formation.

Key words: Diphosphonate; cartilage; calcification; matrix.

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Synthetic diphosphonates have attracted much attention in recent research on biologic calcification. These compounds contain the P-C-P linkage and are thus related to inorganic pyrophosphate (P-O-P). Both have a marked *in vitro* effect on calcium hydroxyapatite growth and dissolution (for reviews see *Russell & Fleisch* 1970, *Russell & Smith* 1973, *Fleisch et al.* 1973). Unlike pyrophosphate substances containing the P-C-P bond cannot be hydrolysed biologically. As predict

ed on the basis of *in vitro* studies, diphosphonates such as disodium etidronate (disodium ethane-1 hydroxy 1 1-diphosphonate, EHDP) interfere *in vivo* with bone destruction as well as with mineralization (*King et al* 1971 *Fleisch et al* 1973 *Larsson* & *Larsson* 1973, *Russell et al* 1973)

At the light microscopic level, high doses of EHDP given to experimental animals (10–60 mg/kg body weight/day) result in the formation of wide osteoid seams and lack of cartilage mineralization in the epiphyseal growth plate (*King et al.* 1971 *Russell et al.* 1973 *Schenk et al.* 1973) However the postulated isolated effects on growth and dissolution of apatite crystal have become a matter of some dispute It has also been suggested that EHDP interferes with alkaline phosphatase (*Höltgens et al* 1973) membrane transport of calcium (*Guilland et al.* 1974) collagen fibrillogenesis (*Larsson* 1974a) and also with glycosaminoglycan synthesis in the epiphyseal growth plate (*Larsson* 1975)

The critical blood concentration at which EHDP will interfere with deposition of crystalline hydroxyapatite has been calculated to correspond to a dose of 5 mg EHDP/kg body weight (*King et al.* 1971) At the ultrastructural level inhibition of crystal growth was observed in forming dentin at low doses of EHDP (2–10 mg/kg body weight *Larsson* & *Larsson* 1973) A complete lack of crystal formation was seen at higher doses of EHDP (30–40 mg/kg body weight, *Larsson* 1974a) The main purpose of these studies was to test the validity of EHDP as a specific inhibitor of apatite crystal formation Such a specific effect was, however not seen in the forming dentin (*Larsson* 1974a) It is the aim of the present investigation to give a report on the light microscopic and ultrastructural changes in the epiphyseal growth plate of young rats given high doses of EHDP To our knowledge, such changes have not yet been investigated at the subcellular level

MATERIAL AND METHODS

One-day old Sprague-Dawley rats, weighing approx. 5 g, were injected intraperitoneally with 0.05 cc f a physiological saline solution containing EHDP* at a concentration of 3000 or 4000 μg/cc, corresponding to 30 and 40 mg EHDP/kg body weight/injection. One group of rats was given total of four injections with a 20-hr interval between the first two injections and a 12-hr interval between the following injections. Another group of animals was given two injections of EHDP at a conc. of 4000 μg/cc. These injections were given with a 15-hr interval. Control animals were either injected with the same olume of physiological saline or were left untreated. There were three rats in each of the experimental groups and in the control group.

The rats given four injections of EHDP were killed six hrs after the last injection, the rats given two injections being killed 32 hrs after the last. The rats were anaesthetized with Mebumal and perfused for a short time through their left cardiac ventricle with 0 1 per cent Prokain-HCl (*Ferrosan*

* A gift from the Procter and Gamble Company Cincinnati, USA

Figs. 1–4 are from normal control animals. Fig. 1 is a light microscopic picture. Figs. 2–4 are electron micrographs.

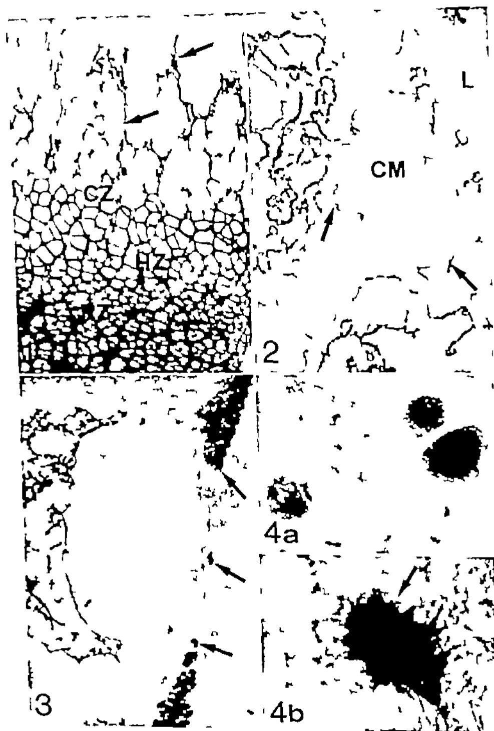
Fig 1 Light microscopic picture illustrating the epiphyseal-metaphyseal complex in the rat tibia. The following zones are identified PZ = proliferative zone, HZ = hypertrophic zone, CZ = zone of provisional calcification. Arrows = calcified trabeculae in the metaphysis. Toluidine blue stain, × 100

Fig 2 In the enlarged chondrocyte lacunae (L) of the hypertrophic zone numerous cytoplasmic processes (or cell remnants) extend towards the uncalcified cartilage matrix (CM) × 2200

Fig 3 The longitudinal septa start calcifying in the zone of provisional calcification. Roundish clusters of crystals are observed in the matrix of the septa (arrows) × 4200

Fig 4a. Matrix vesicles at high magnification. The matrix of the vesicles is homogeneous and amorphous and the esicles are enclosed by triple-l yered membrane. × 118000.

Fig 4b Apatite crystal formation starts within the matrix vesicles. Calcification of the surrounding matrix commences upon growth of the crystals through the membrane of the esicles. Subsequently the vesicles become embedded in clusters of apatite. Arrow points at still isibl triplelayered membrane of vesicle almost obscured by apatite crystals. × 118000


GZ
HZ
2
L
CM
3
4a
4b

et al. 1967) in 0.05 M cacodylate buffer (pH 7.2) containing 0.2 M sucrose. Immediately thereafter the animals received a second perfusion for several minutes of ? per cent glutaraldehyde (Polysciences Inc., Warrington, Pennsylvania, USA) in the same buffer this time containing 2 per cent Dextran (mwt 20 000). Fixation of excised tibial epiphyses was continued at 4 C in 4 *per cent* glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2–4 hrs. Rinsing, postfixation, embedding in Epon and thick and thin sectioning was performed as described *previously* (*Larsson* 1974*a*). The thick sections were stained with toluidine blue. The ultrathin sections were contrasted with uranyl-lead or left uncontrasted and examined in a Philips EM 300 electron microscope at 60 or 80 kV.

RESULTS

1 *Controls*

The morphology of the epiphyseal growth plate in the rat is well documented in many reports in which the general light microscopic (Fig. 1) and subcellular features are described. The ultrastructure of the intercellular septa in hypertrophic and calcifying zones of the normal control animals is illustrated in Figs. 2–4. The below description is mainly concerned with the induced ultrastructural changes of the septa observed in the EHDP treated rats while the *cellular effects will be* reported elsewhere.

2. *EHDP Treated Animals*

A. *Light microscopic findings.* Obvious *changes were observed in all three experi*mental groups. Each one of the rats receiving four injections of 30 mg EHDP/kg body weight exhibited increased width of the hypertrophic zone (Fig. 5). In the zone of provisional calcification, most cartilage septa seemed to be uncalcified. However in areas where cells of an atypical appearance were observed (Figs. 5, 6) the septa were calcified. In the metaphysis, the calcified septa were fewer and thinner than in the controls *and uncalcified osteoid and cartilage was also* found in this region (Fig. 6).

In the metaphysis of rats receiving four injections of 40 mg EHDP very thin and poorly developed calcified septa were observed. Furthermore, there was no evident calcifica*tion of the* cartilage septa in the zone of provisional calcification. However in regions where atypical cells were observed, calcified septa similar to those mentioned above (Figs. 5, 6) were present.

In rats receiving two injections of 40 mg EHDP/kg body weight each small "islands" of weakly stained cartilage or osteoid were *found in the* metaphysis (Fig. 7). These were separated from the zone of provisional calcification by an area where calcified septa were almost lacking. Calcified cartilage septa were present, however in the zone of provisional calcification. The hypertrophic zone was not clearly widened in this group. Toluidine blue stained material was present in the lacunae *where cell remnants are not normally* identified (cf. Fig. 1). Granules similar to those normally seen in the proliferative zone were observed within the lacunae (Fig. 7).

B. *Ultrastructural findings.* Rats receiving four injections of 30 mg EHDP exhibited uncalcified cartilage septa and there was no longer any evidence of a normal zone of pro*visional calcification. In these* septa matrix vesicles were observed more frequently than in septa in the controls (Fig. 8). Crystals were only occasionally seen within these vesicles. The matrix of the septa was also more densely-textured than that in corresponding areas in the controls. This seemed, at least partly, to be due to the presence of deposits of a coarsely-textured material (Fig. 8 inset). There were no structures in the controls corresponding to this material. No direct relationship between *this material* and the vesicles could be recognized.

In rats receiving four injections of 40 mg EHDP many vesicles containing only *single* apatite crystals were visible in the area which might be presumed to constitute the zone of provisional calcification under normal conditions. Again, the texture of the septal matrix was denser than that in the controls. However the coarsely-textured material to be observed after injection of 30 mg EHDP was *not an equally prominent* feature after injection of 40 mg EHDP/kg body weight.



Figs. 5–10 are from EHDP treated animals.
Figs. 5–7 are light microscopic pictures, figs. 8–10 are electron micrographs.

Fig. 5. From a rat given four injections of 30 mg EHDP/kg body weight/injection. The hypertrophic zone has become widened. In the zone of provisional calcification, areas without visible mineralization (arrows) intermingle with areas of calcified septa. In the latter areas, atypical chondrocytes are present (double arrows). Normally the chondrocyte lacunae look empty in this area (cf Fig. 1) but are now filled with cellular remnants. Notice also the lack of calcified septa in the metaphyseal region (M) × 80.

Fig. 6. From a rat given four injections of 40 mg EHDP/kg body weight/injection. Higher magnification of area labelled "M" in Fig. 5. Uncalcified osteoid and cartilage is present at the epiphyseal-metaphyseal border (arrows). Notice lacunae filled with cellular remnants (atypical chondrocytes) × 160.

Fig. 7. From a rat given two injections of 40 mg EHDP/kg body weight/injection. Island (I) of uncalcified osteoid or cartilage in the metaphyseal region is seen to be separated from the epiphysis by zone lacking calcified trabeculae. Atypical chondrocytes are seen in the epiphysis. Toluidine-blue stained granules similar to those normally seen in the proliferative zone of normal control animals are present at the periphery of the lacunae (arrows) × 270.

The density of the matrix in the latter group of animals was mainly due to an increased amount of fine precipitates in the ground substance. Micrographs at higher magnification revealed the presence of a fine meshwork consisting of irregularly shaped non-membranous granules at the intersections of fine fibrils (Fig. 9). Similar but less numerous granules were also seen in the calcifying septae of controls.

As regards rats receiving two injections of 40 mg EHDP/kg body weight each, the interest was focused on the "islands" of uncalcified osteoid or cartilage in the metaphysis (cf Fig. 7). Degenerating chondrocytes were seen centrally while the periphery of the islands was covered with osteoblast-like cells. There was no evidence of calcification of the abundant matrix except for the appearance of apatite crystals within matrix vesicles (Fig



Fig 8 This is from a rat given four injections of 30 mg EHDP/kg body weight/injection. The area depicted is that where calcification of the septal matrix normally would be expected to occur. The number of matrix vesicles seems to be higher than that at any level in normal controls. The matrix exhibits an increased density as compared to matrix with amorphous ground substance in normal control *animals. L = lacunae.* × *15500*

Inset Higher magnification of an area of the septal matrix illustrated in Fig 8. Apart from vesicles and fine fibrils, masses of a coarsely textured material is also present (arrow). The material is not directly associated with the vesicles. × 60000.

10). The appearance of these vesicles was identical to that of vesicles found in the hypertrophic zone of the normal control animals. They were also similar to those observed *in the other two experimental groups* described above. Collagen fibrils were present in the matrix of the islands (Fig. 10). Their number was higher than that otherwise observed in hypertrophic and calcifying zones. Fibrils were most numerous in the region adjacent to the osteoblast like cells. In the area surrounding the degenerating chondrocytes, fibrils were less numerous and the matrix was of an appearance very similar to that observed in the above described group of animals which received *4* × *40 mg EHDP*

The lacunar granules which by light microscopy were observed in the zone of provisional calcification will be the subject of further studies.

DISCUSSION

There is still some dispute about the cellular activity and the detailed subcellular characteristics of the epiphyseal growth plate. The concept that degenerating hypertrophic chondrocytes ultimately die has recently been questionned by *Holtrop* (1972) who found evidence of a cell activity beyond the zone of provisional calcification. When testing the potential of EHDP as a specific *in vivo* inhibitor *of biologic calcification*, an overall under



Fig. 9 From a rat given four injections of 40 mg EHDP/kg body weight/injection. The matrix of the septa contains vesicles and fibrils. There is also an accumulation of small granular deposits of an amorphous material connected to the fine fibrils (arrows, V = vesicles) × 96000

standing of the normal dynamics of the experimental tissue is a prerequisite in order that the results may be more accurately interpreted. With respect to the calcification process *per se* extracellular matrix vesicles or globules have attracted much interest in recent research. These vesicles appear mainly in the longitudinal septa of the hypertrophic zone and they have been ascribed an infinite role in the calcification process by initiating apatite crystal formation (*Anderson* 1969 1973 *Bonucci* 1970 *Thyberg & Friberg* 1970 *Alcock* 1972). The vesicles exhibit alkaline phosphatase and ATP-ase activity (*Matsuzawa & Anderson* 1971). It has also been proposed that some vesicles, due to acid phosphatase activity would be of lysosomal nature (*Thyberg & Friberg* 1970). Globules similar to those of the epiphyseal septa are also present in early dentin formation. Alkaline glycerophosphatase as well as alkaline pyrophosphate-phosphohydrolytic activity at the subcellular level in these globules have been illustrated (*Larsson* 1973 1974b). There is good reason to believe that the vesicles-globules in calcifying cartilage and predentin play a role in the initiation of the calcification process. Consequently this system is of great interest in studies of the specific inhibition of mineralization. Inhibition of apatite formation in globules-vesicles would be expected to result in a lack of mineralization also of the surrounding matrix. Further evidence for such a concept was produced by *Simon et al* (1973)

Lack of apatite crystals was a prominent feature in the EHDP treated rats in the present investigation. Mineralization was inhibited in matrix vesicles as well as in the intercellular cartilaginous matrix where a presence of crystals normally is to be expected. Formation of crystalline hydroxyapatite has been proposed to be preceded by the formation of an amorphous precursor (see *Termine* 1972)



Fig 10 From the border of an uncalcified cartilage or osteoid "island" in an animal given two injections of 40 mg EHDP/kg body weight/injection (cf Fig. 7) Osteoblast-like cells (0) cover the periphery and an abundance of small roundish bodies can be seen in the matrix.
The area nearest to the cells is rich in collagen fibrils Some of these seem to emanate from the surface of the cells (arrows) Clusters of apatite crystals are seen, but only within the vesicles in the matrix (double arrows) × 34000

If the primary action of EHDP is to prevent the transformation of amorphous calcium-phosphate to crystalline an accumulation of amorphous salt in the EHDP treated epiphyseal plates would be expected. Still an adequate method by which to preserve this salt in routine electron microscopy is not available and, hence, our understanding of the morphology of the salt remains inadequate (*Termine* 1972) The coarsely textured material which in the present study is found to be localized in the matrix of the septa could represent such a salt. Alternatively the material might represent remnants of ruptured vesicles.

Fine precipitates were also observed in uncalcified predentin in EHDP treated rats (*Larsson* 1974a) The morphology of the precipitates which in the present study were seen in the calcifying zone of the epiphyseal septae (cf Fig 9) resembles closely the proteoglycan granules described by *Eisenstein et al.* (1973) and *Thyberg et al.* (1973) These authors stated that these granules were most numerous in the proliferative and hypertrophic zones while they decrease distinctly in number in the calcifying zone which is in agreement with biochemical data reported by *Larsson* (1973) Our observation of an increased number of such granules in the calcifying zone in rats treated with high doses of EHDP might therefore indicate an inhibited degradation of proteoglycans by EHDP Evidence of an EHDP inhibited synthesis of matrix glycosaminoglycans in rabbit epiphyseal growth plate *in vivo* has recently been presented (*Larsson* 1975) It has been suggested by several authors that a degradation of the macromolecular proteoglycan complexes might be an integral part of the calcification

process (*Campo* & *Tourtelotte* 1967 *Bowness* 1968, *Howell et al.* 1969). The present observations suggest that EHDP might interfere with calcification also at this level besides its specific inhibitory effect on apatite crystal formation *in vivo* as previously reported (*Larsson* & *Larsson* 1973).

Transport of calcium and phosphate to the matrix vesicles has been considered a prerequisite for the formation of crystalline calcium phosphate in these subcellular constituents. So far the mechanism of such a transport is unknown. With respect to the effect of EHDP the results obtained by *Guilland et al.* (1974) may be of significance. These authors found evidence in support of the assumption that EHDP may interfere with membrane transport of calcium. Such an interference, perhaps also with the membranes within the matrix vesicles, would be expected to result in a lack of crystal formation. As regards phosphate, the idea has been advanced that an alkaline phosphatase or an ATPase would control phosphate transport to the vesicles (*Anderson* 1973). In an excellent review *Alcock* (1972) summarized the current concepts of cartilage calcification, emphasizing in particular the role of alkaline inorganic pyrophosphatase (PPase) and matrix vesicles. Evidence has been presented that a PP phosphohydrolytic enzyme is associated with the membranes of the dentinal globules (*Larsson* 1974b). *Hilgers et al.* (1973) demonstrated the competetive inhibition of a PPase from hamster molars by EHDP. Therefore, if the function of such a PPase were to supply the globules-vesicles with phosphate compounds for the initial formation of apatite it might be expected that administration of EHDP could interfere severely with this process. At present, however it seems hardly possible to single out any specific factor by which the changes observed in EHDP treated animals might be explained.

Matrix vesicles are continuously formed in the epiphyseal growth plate and it is obvious from our findings that EHDP does not interfere with this cellular process. Our observation in EHDP treated animals that the number of vesicles is increased in the zone where calcification normally is to be expected together with the finding of an accumulation of hypertrophic chondrocytes lend support to the hypothesis that the matrix vesicles are produced by living cells and do not represent remnants from dying cells. The prolonged persistence of these cells induced by EHDP could be due to an inhibited mineralization of the septa and a disturbed cell migration. Since the vesicles no longer become embedded in crystalline apatite in the surrounding uncalcified matrix, the resultant accumulation of vesicles could give the false impression of an increased total number of vesicles formed by the chondrocytes.

Schenk et al. (1973) found mineralization of the epiphyseal cartilage to be inhibited within the first few hours after each of several injections of EHDP. This was followed by a recovery period that resulted in step-like delineations which marked a temporary arrest of mineralization. In "an inhibition of bone mineralization and recovery study" *King et al.* (1971) reported massive amounts of osteoid tissue in rats given 50 mg EHDP/kg body weight/day for 28 days. These lesions healed within a 3 to 6 month recovery period, only minor remnants of osteoid being left uncalcified in the tissues. When EHDP is administered for longer periods of time, an interference with vitamin D metabolism must also be considered, at least if EHDP is given in high doses (*Baxter et al.* 1974). *King et al.* (1971) considered the mechanism of recovery from a standpoint of diffusion. "Bleeding of EHDP" from the osteoid and calcified borders would permit calcification to start again. In the study by *Schenk et al.* (1973) mineralization was proposed to resume as the concentration of EHDP fell in the intercellular fluid prior to the subsequent injection. The half life of EHDP ^{14}C in bone was calculated at 30 days by *King et al.* (1971) and at 12 days by *Michael et al.* (1972). The results of the cited studies clearly indicate that the injection of EHDP will cause only a temporary inhibition of the epiphyseal cartilage

mineralization. Mineralization will resume upon withdrawal of the drug This is also supported by our above described findings in the group of animals which were given two injections of 40 mg EHDP/kg body weight each and killed 32 hours after the last injection. In these animals, remnants of uncalcified cartilage were identified in the metaphysis. However at the periphery of these remnants, large amounts of collagen were present, and there was also evidence of crystal formation in the vesicles. The presence of collagen is not surprising This is the zone where osteoid formation normally takes place. The appearance of crystals in the vesicles would seem to indicate a resumed mineralization. The fact that similar uncalcified "islands" were not observed in the other two groups of experimental animals killed 6 hours after the last injection of EHDP is not easily explained. It could indicate that a period longer than 6 hours is required for such matrix "islands" to form in the metaphysis in the presence of EHDP It could also be related to an inhibited synthesis of matrix glycosaminoglycans (*Larsson* 1975) which could also explain the lack of toluidine-blue staining observed in the "islands"

The collected data about the effects of EHDP seem to indicate that EHDP acts at a level where the initial deposition of crystalline calciumphosphate normally is expected to take place The question of a possible cellular effect needs further investigation. The atypical cells observed in the present investigation could represent either hypertrophic chondrocytes deleteriously affected by EHDP or "reactivated chondrocytes at a stage of transformation into bone cells (see also *Holtrop* 1972) If so EHDP in the doses used in the present study would have a much more complex effect on calcifying tissues than hitherto anticipated.

We are grateful for financial support for this work from the *Swedish Medic l R search Cou cil* (Project nr 24X 3798) and *King Gustaf the Fifth Eightieth Birthday Fund* The a thors are also grat ful to the Dept. of Pathology Dalhousie University Halifax, N.S Canada for the use of electron microscopic facilities for part of this investigation.

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RENIN INACTIVATION *IN VITRO* IN NON PREGNANT RABBIT UTERINE AND KIDNEY TISSUE

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Jorgensen, J Renin inactivation *in vitro* in non-pregnant rabbit uterine and kidney tissue Acta path. microbiol. scand. Sect. A, 84 28-32, 1976.

In slices of non-pregnant rabbit uterus, renin was gradually inactivated during incubation *in vitro* at 37 C, for 44 hours. The percentual rate of inactivation (40 per cent/44 hours) was, however only about half of that found in uterine slices from pregnant and post-partum animals despite the fact that the endogenous renin concentration was much lower in the non pregnant uteri. In control experiments with kidneys with the same high renin content as in the pregnant uteri only about 30 per cent of the renin was inactivated in 44 hours.

Key words Renin inactivation uterus kidney

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In a previous paper it was demonstrated that slices of pregnant and post partum uterus incubated *in vitro* had a considerable ability to inactivate their endogenous renin content (*Jorgensen* 1974 b) In slices of renal cortex only a slow decrease in renin was seen.

The aim of the present study was to investigate whether or not a similar inactivation of renin took place during incubation of normal non-pregnant uterine and kidney tissue (Part I) The ability of tissue renin to diffuse into the incubation medium was also studied (Part II) The study includes a few experiments with similar incubation of tissue from pregnant rabbits or taken shortly post partum.

MATERIALS AND METHODS

Animals Uterine wall and renal cortex from 12 non-pregnant, 2 pregnant (26th and 27th day of pregnancy) and 3 post-partum (about 11 12 and 22 hours after delivery) albino country rabbits from the State Serum Institute were used. In most *cases the animals were* anaesthetized and the uterus and kidney removed by an aseptic operation as previously described (2)

The whole supravaginal part of the uterus was used when the animals were non pregnant, otherwise an antimesometrial strip was used.

In a few cases non-pregnant rabbits were sacrificed by a blow on the neck, uterus and kidney were removed without aseptic precautions, and the kidneys kept for 45 min at 4 C before use. The whole supravaginal part of the organ was utilized and tissue from two animals was used in each experiment to give more material. Bacteriological investigation of incubation medium from these preparations incubated for 0 or 44 hours showed no bacterial growth.

Preparation and incubation of tissue slices in

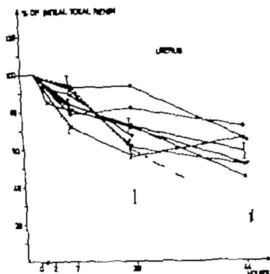


Fig 1 The in vitro inactivation of uterine renin. The decrease with time of total renin (slices plus medium) in tissue from non-pregnant animals (solid lines) individual experiments. Mean values from non-pregnant (solid) pregnant (dashed) and post-partum experiments (dotted lines) are also given. The bars indicate 1 SEM.

vitro was performed as previously described (2) except for the following modifications.

In most cases 16 test tubes were prepared from each organ and incubated at 37 C pH 7 4 for 0 7 20 and 44 hours in quadruplicate in incubation tubes measuring 3 cm in diameter After incubation the samples were placed in an ice-bath, the pH of the incubation medium was measured and pH adjusted to 6.5–7.0 with 2 N HCl. The four samples of each incubation period were pooled and separated in *incubation medium* and tissue by a centrifugation at 3000 g for 10 min at 4 C. Some preparations of non-pregnant uterus and kidney were only incubated in triplicate, and in some cases only for 0, ½, 2, 7 and 20 hours.

pH in the incubation medium after incubation remained stable t about pH 7 (6.9–7 4) I one case pH decreased to values of about 6.2.

Renin extraction In most cases the tissue was frozen and thawed 3 times and then homogenized in Potter Elvehjem homogenizer after addition of 4 ml phosphate buffer (0.01 M pH 7 4) In some experiments the tissue as previously was homogenized after addition of pyrophosphate buffer (0.02 M pH 5.5) Extraction at pH 7 4 and pH 5 5 gave identical results (N = 12) After 18 hours at 4 C, the samples were centrifuged at 3000 g, and the supernatants stored t – 20° C until assay

Renin radioimmunoassay was performed as previously described (*Poulsen & Jorgensen* 1974) The renin concentration was expressed in Goldblatt units (GU) by reference to a purified hog renin standard preparation, kindly supplied by Dr Haas and identical with the preparation donated by Dr Haas to the National Institute for Biological Standards and Control, Holly Hill, London.

Calculation of Renin Content in Tissue and Incubation Medium

The total renin content was calculated as the sum of renin in the present tissue slices and the incubation medium and expressed in GU per g tissue.

Tissue slices the renin concentration in the supernatant after homogenization was multiplied by the dilution factor of homogenization and divided by the weight of the tissue to give the content in GU per g tissue.

Incubation medium: the renin concentration was determined and the amount calculated after measuring the volume by weighing. The amount was divided by the number of g tissue which was previously present in the samples.

RESULTS

Part I The Total Renin Content in Slices plus Incubation Medium during Incubation

a) *Uterus* In the *non pregnant* uterus there was a decrease in enzymatically active renin from the start of the experiments continuing for the whole 44 hour incubation period (Fig. 1) After 7 and 20 hours there was a decrease to a mean value of about 80 and 70 per cent, respectively of the initial amounts. After 44 hours it had decreased to about 60 per cent. One of the 8 experiments differed from the others, showing an increase to 170 per cent of the initial amount after 20 hours incubation. The initial renin concentration varied between 0.5 and 3.2 (mean 1.5) GU/g tissue. These differences had no apparent influence on the relative decrease in renin.

A few experiments were performed in *pregnant* uterus. No loss of renin was observed after the first 7 hours of incubation. Upon further incubation the renin content decreased to about 80 and 30 per cent of the initial amounts after 20 and 44 hours of incubation, respectively This decrease in renin was identical to, although slightly slower than in the previous analogous experiments.

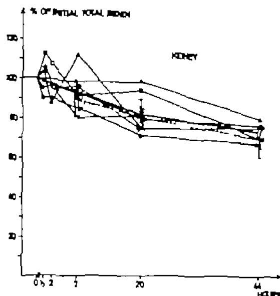


Fig 2 The in vitro inactivation of kidney renin. The decrease with time of total renin (slices plus medium) Symbols as in Fig. 1

The mean values of all experiments (N = 7 uteri removed between the 26th and 31st day of pregnancy) (previous and present) are given in Fig 1 The initial renin concentrations were 12–34 (mean 24) GU/g tissue.

The experiments were also repeated with post partum uterus. There was a decrease in renin as previously found in all cases from the start of the experiments and continuing with a rather constant rate for the first 20 hours, after which the rate decreased. After 7 20 and 44 hours there was a decrease in renin to about 75 40 and 25 per cent respectively of the initial amounts. The mean values of all *experiments are given in Fig 1* (N = 7 uteri removed between 10 and 33 hours post-partum) The initial renin concentrations were 3 6–33 GU/g tissue (mean 14)

b) *Kidney cortex* In 7 kidneys from non-pregnant animals there was a slow decrease in total renin during the 44 hours of incubation to about 75 per cent (Fig. 2) This is identical to the decrease seen in the previous and 5 additional experiments using kidneys *from pregnant (N = 5) and post* partum (N = 5) animals. The mean values are given in Fig. 2. The initial renin concentrations varied between 8 and 27 (mean 16) GU/g tissue.

Part II Distribution of Renin between Tissue and Incubated Medium

a) *Uterus* Using *non pregnant uterus* the total renin *in the medium* was 0 1–0.2 and in one case 0.5 GU/g tissue at the start of the experiments. This increased slightly to about 0 4–0 6 *GU/g tissue at 44* hours with most of the increase occurring during the first 7 hours of incubation (N = 7)

Although the amount of renin present in the medium was remarkably stable throughout the incubation, the fraction thus composed of the total amount was, however increasing from 7–17 *per cent to 40–85 per cent at the* end of the 44 hour incubation. The mean value of all experiments is shown in Fig. 3 Thus, the decrease in the total renin content (Fig 1) was caused by a decrease in the renin content in the tissue slices.

Using *pregnant uterus* the amount of renin present in the incubation medium was also constant during the incubation with values between 0.8 and 1 6 GU/g tissue The relative amounts of renin present in the medium also did increase in these during incubation. They were initially (N = 5) about 8 per cent and at 44 hours of incubation the medium contained 13–65 per cent of the total renin content (N = 3) The mean value of all experiments is shown in Fig 3

Using *post partum uterus* identical results *were obtained.* The renin content in the medium was rather constant during the incubation with values between 0.5 and 1.6 GU/g tissue. The relative amounts of renin rose gradually from 5–20 per cent to 20–100 per cent at 44 hours (Fig 3) of incubation (N = 7)

b) *Kidney* The renin content of the incubation medium in all preparations from all states of pregnancy increased gradually during incubation from 0.2 GU/g tissue at the beginning of the incubation to values between 1.5 and 10 GU/g tissue after 44 hours of incubation. The relative amounts of renin in

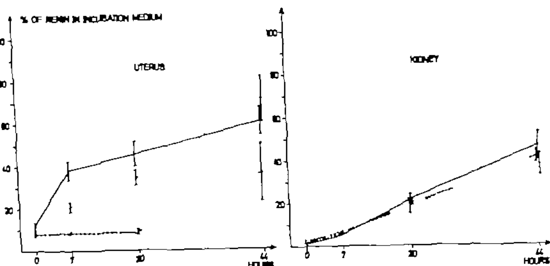


Fig 3 Renin in the incubation medium. The relativ amount of renin present in the incubation medium with time for experiments with uterus (left) and kidneys (right) Mean values are given from non-pregnant (solid) pregnant (dashed) and post-partum experiments (dotted lines) The bars indicate 1 SEM.

the incubation medium increased gradually from about 1 per cent of the total to 25–60 per cent during the 44-hour incubation (Fig 3) (N = 6, 4 and 5 using kidney from non pregnant, pregnant and post-partum animals, respectively)

DISCUSSION

In previous papers it was demonstrated that the rapid and pronounced fall of rabbit uterine renin which occurs in vivo during the immedi ate post partum period could not be explained by a release of the renin to the blood stream. It was further demonstrated that pregnant and post partum rabbit uterus had a considerable ability of their own to inactivate renin during an incubation *in vitro* (1 2) In the present study this *in vitro* inactivation of renin was also demonstrated in normal non-pregnant uterine and kidney tissue.

The concentration of renin in the pregnant uterus is far higher than in the non-pregnant uterus. If the inactivating enzyme system had the same capacity in these two conditions one would expect the relative rate of inactivation to be equal to or higher for the lowest renin concentrations. The opposite was found, how ever The relative rate as well as the absolute amount of renin inactivated was highest in the pregnant and post-partum uterus with the highest renin concentrations. This indicates that the inactivation mechanism was stimulated in this condition. It should be remembered, however that any rate of inactivation seen in this type of experiment may be the result of a simultaneous generation and inactivation of renin.

This work was supported by grants from the *Danish Medical Research Council, Fonden til Lægevidenskabens Fremme* and *Carl and Ellen Hertz Legat* (to Jørgen Jørgensen) and *King Christian X Foundation* (to Jens Bing) Valuable gifts of Estilbin and Follicyclin from Dumex and CIBA, respectively are gratefully acknowledged.

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5 *Poulsen K & Jorgensen, J:* An easy radioimmunological micro-assay of renin activity concentration and substrate in human and animal plasma and tissues based on angiotensin I trapping by antibody J Clin. Endocr 39 816–825 1974

Acta path. microbiol. scand. Sect. A, 84 33–39 1976

ENZYME HISTOCHEMICAL STUDIES OF RABBIT BILE DUCTS WITH AND WITHOUT BILE FLOW

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Simert, G., Hammar, E., Hansson, J. A., Hägerstrand, I. & Vang, J. Enzyme histochemical studies of rabbit bile ducts with and without bile flow. Acta path. microbiol. scand. Sect. A 84 33–39 1976.

The histochemical enzyme pattern in normal, extra-hepatic bile ducts from rabbits was studied. A difference between the duct epithelium and the crypt epithelium was noted mainly in the activity of gamma-glutamyl-transpeptidase and alkaline phosphatase which only stained positively in the crypts. No difference from the normal enzyme pattern was noted after diversion of the bile flow for up to 30 days.

Key words: Bile ducts; bile flow; enzyme histochemistry.

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The function of the extra-hepatic bile ducts other than the obvious function as bile carrying ducts is largely unknown. Histological studies of their structure are few and in most cases old and the enzyme histochemistry seems to have been investigated only occasionally. The effects of diversion of the bile flow on the structure of the common duct have not previously been studied by enzyme histochemical techniques.

In the present investigation, the pattern of 10 enzymes in walls of extra hepatic bile ducts in the rabbit has been studied. With a view to investigating whether the bile had any influence on the enzyme pattern, the ducts in a separate group of rabbits were deprived of bile by diversion of the bile flow. The enzyme pattern was studied histochemically and compared with that of the bile carrying ducts.

MATERIAL AND METHODS

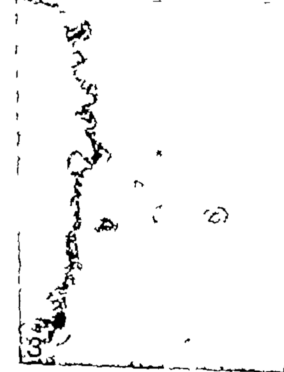
Eleven young rabbits weighing [illegible],000–3 000 grams were used. They were kept for a fortnight in our animal house for observation and adaption before use. The animals were divided into two groups. *A*: rabbits used for the study of the enzyme pattern of bile-carrying ducts and *B*: rabbits used for the study of the duct walls after bile diversion. *A*: The rabbits in this group (N = 4) were fasted overnight. The animals were sacrificed, using an overdose of mebumal sodium, and the common bile duct was removed and frozen in liquified natural gas and stored at −70° C for later preparation. *B*: These rabbits (N = 7) were fasted overnight. In the morning they were anaesthetized with mebumal sodium. An upper midline incision was made and the common duct was carefully dissected from the surrounding tissue. A silk ligature was tied

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3 *Poulsen K & Jorgensen, J* An easy radioimmunological micro-assay of renin activity concentration and substrate in human and animal plasma and tissues based on angiotensin I trapping by antibody J Clin. Endocr 39 816–825 1974




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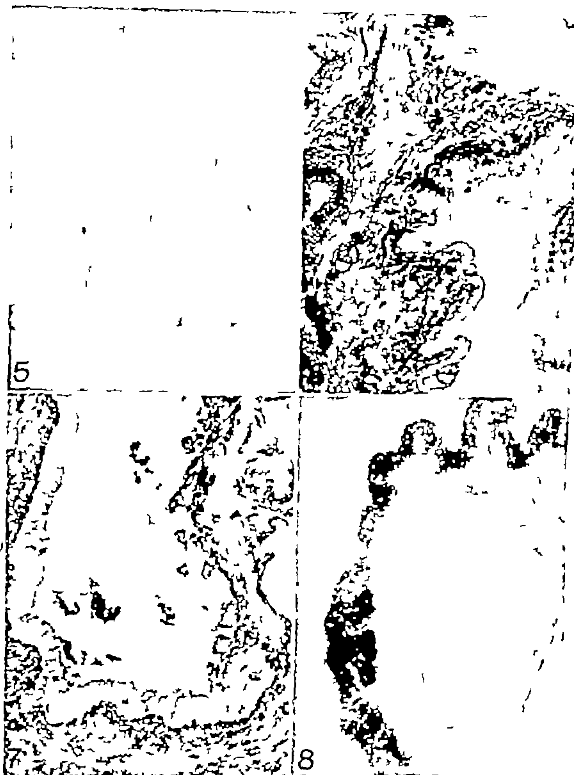


Fig 5 Acid phosphatase. Very weak activity in th epithelium. × 400
Fig 6 ATP ase. Strong subepithelial activity and cased wall acti ity × 100
Fig 7 Leucine aminopeptidase. Strong subepithelial acti ty × 160.
Fig 8 Non-specific esterase. Strong cytoplasmatic epithelial activity × 160.

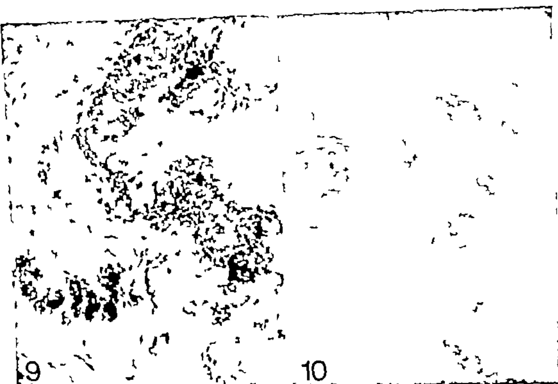


Fig 9 Alkaline phosphatase. Strong subepithelial activity + activity in the lumen of the crypts. × 160.
Fig 10 Mono-amino-oxidase. Very weak activity situated mainly in the vessel walls × 160.

Adenosine triphosphatase displays a continuous border activity in the epithelium near the lumen. There is very strong subepithelial activity in the basal membrane and also in the stromal vessel walls.

Leucine aminopeptidase shows no activity in the epithelium. There is strong activity in a narrow subepithelial zone corresponding to the basal membrane. There is also moderate activity in the stromal vessel walls and in the collagenous connective tissue cells.

Non specific esterase shows even cytoplasmatic activity in the epithelial cells and is everywhere well-pronounced. There is no activity elsewhere in the wall.

Beta glucuronidase demonstrates moderately pronounced activity in the cytoplasm of the epithelium. It is more concentrated api

TABLE 1 *Enzyme Activities in the Common Bile Duct of the Normal Rabbit*

	Epithelium	Crypts	Wall
Alkaline Phosphatase	—	±	+ + +
Adenosin Triphosphatase	±	±	+ + +
Leucine Aminopeptidase	—	—	+ +
Gamma Glutamyl-Trans-Peptidase	—	±	—
Acid Phosphatase	±	±	—
Beta-glucuronidase	+ +	+ +	—
Non-Specific Esterase	+ + +	+ + +	—
Lactic Dehydrogenase	+ +	+ (+)	±
Succinic Dehydrogenase	+ + +	+ + +	±
Mono-Amino-Oxidase	(+)	(+)	—

cally. Otherwise there is no activity in the wall.

Succinic dehydrogenase shows very strong activity in the epithelium. In addition only moderate activity in the vessel walls is noted.

Mono-amino-oxidase displays very weak activity in the epithelium and insignificant activity in the vessel walls.

Gamma glutamyl transpeptidase activity can be seen in the cytoplasm of the epithelium of a moderate number of the deep crypts. In the rest of the epithelium and in the other parts of the wall there is no activity.

Lactic dehydrogenase demonstrates even cytoplasmatic activity in the epithelium. A certain increase in activity towards the transition to duodenal mucosa is noted.

Effect of Bile Flow Deviation

After 24 hours a distinct infiltration especially of the outer parts of the common duct wall by polymorfonucleated leucocytes, was seen. These showed activity for alkaline phosphatase, beta-glucuronidase and acid phosphatase. This infiltration started to decrease on the tenth day and then disappeared. This course of events is quite clearly a response to the operative trauma. No changes whatsoever in the appearance of the bile duct or the activities of the enzymes below the ligature were detected either in the luminal part or in the crypts even 30 days after the operation.

DISCUSSION

There is distinct evidence that the epithelium of the common bile duct is metabolically very active. The nature of the activity however is largely unknown. *McMinn & Kugler* (1961) were able to show that the bile ducts of mouse, hamster rat, guinea pig cat, dog and rhesus monkey secreted a complex, part of which was mucin. This secretion seemed to originate from the goblet cells as well as from the luminal epithelium and the crypts. *Van der Zypen* (1972) found in electron microscopic studies that the epithelium in the large extra hepatic ducts in man consists of highly active cells and that there are large capillaries immediately under the basal membrane. These capillaries are of a type equipped with numerous pores indicating a highly active exchange process between the epithelium and the vascular system.

A possible difference in the function of the crypts and the luminal epithelium is not clearly defined. The crypts have for a long time been considered to be glands with special secretory functions. This, however could not be confirmed by *McMinn & Kugler* (1961) *Cohen* (1964) using the technique of colchicin arrest, found that the majority of the mitoses of the common bile duct epithelium in rat was found in the crypts. *Chou & Chou* (1973) also showed that the main part of the regeneration of common bile duct epithelium after trauma emanated from the crypts.

The present study shows that there is a distinct difference between the enzyme pattern of the epithelium of the crypts and that of the lumen. In the crypts there is an inconstant activity of gamma-glutamyltranspeptidase and alkaline phosphatase which is completely missing in the luminal epithelium. The location of the activity of the acid phosphatase indicates that at least some of the lysosomes have an apical position in the crypts as compared with that in the luminal cells where the activity of these enzymes is situated more basally. These differences indicate that the epithelium in the crypts may have a function different from that of the epithelium of the lumen. The findings may however merely reflect varying degrees of maturity of the cells.

Only a few histochemical studies of the extra-hepatic bile ducts are available. *McMinn & Kugler* (1961) compared different species with regard to alkaline phosphatase and succinic dehydrogenase. They found succinic dehydrogenase in the epithelial cells of all studied species. Studies of alkaline phosphatase showed species differences and epithelial activity was not observed except in dogs where strong activity both in the luminal

epithelium and in the crypts was noted. The rabbits in the present study show still another enzyme pattern, alkaline phosphatase activity being present only in some of the crypts. These types of species differences are well known from other studies, for instance from studies of the liver To our knowledge, the enzyme pattern in man has not been studied. *Chou & Gibson* (1971) showed the enzyme distribution in rats which corresponds very much to our results. As mentioned above, however there are differences in alkaline phosphatase activity and also in the activity of leucine aminopeptidase which seems to be low in the basal membrane of the rat, in contrast to the high activity in rabbits.

Very few studies have been carried out to ascertain whether absence of bile flow has any effect on the wall of the bile ducts and their functional state. *Hou* (1961) using light microscopy studied the effect of accelerated and reduced bile flow in guinea pigs, but did not find any change in the epithelium corresponding to variations in bile flow *Chou & Chow* (1973) observed no change in healing ability or enzyme pattern following hepatic duct ligation in rats. The present more extensive enzyme study confirms their results. As far as the activity and function of the epithelium of the common bile duct can be studied by enzyme histochemical methods there seems to be no functional or structural change up to at least 30 days after bile flow diversion.

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THE ALVEOLAR LINING LAYER IN EXPERIMENTAL PARAQUAT POISONING

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Robertson, B., Grossmann, G & Ivemark, B The alveolar lining layer in experimental paraquat poisoning Acta path. microbiol. scand. Sect. A, 84 40–46, 1976

Rats were injected with paraquat, 35 mg/kg, and killed 8 or 12 hours later Electron microscopic studies of the lungs revealed degenerative changes in alveolar epithelium and alveolar capillary endothelium after 8 hours. After 12 hours, the alveolar lining layer was contaminated with cellular debris from disintegrating alveolar epithelium. This contamination is probably responsible for the inactivation of lung surfactant that can be demonstrated in early stages of experimental paraquat poisoning

Key words Alveolar lining layer paraquat poisoning lung surfactant.

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The noxious effect of paraquat (dimethylbipyridylium dichloride) on the mammalian lung has been documented *in several reports* (for review see 10)

In the rat, a single subcutaneous injection of paraquat, 35 mg/kg, leads to necrosis of the alveolar epithelium and the alveolar capillary endothelium (8) As the destructive process involves the granular pneumocytes, it naturally interferes with the synthesis of pulmonary surfactant lecithin (6) Accordingly there is a gradual exhaustion of the endogenous supply of pulmonary "surfactant" with consequent alveolar collapse. However animal experiments have revealed that inactivation of the pulmonary surfactant system takes place already within a few hours after paraquat injection (11) This early effect can hardly be explained by consumption of the surfactant pool in the alveolar lining layer Therefore, some additional mechanism must be operating

The present study was undertaken in order to make out whether or not ultrastructural abnormalities of the alveolar lining layer could offer some explanation of the early inactivation of pulmonary surfactant in experimental paraquat poisoning

MATERIAL AND METHODS

Preliminary Series
Surface Properties of Alveolar Wash

In a preliminary experimental series, adult female Sprague-Dawley rats were *killed* 2–24 hours after subcutaneous injection of paraquat, 35 mg/kg The lungs were washed with 10 cc saline by means of a syringe affixed in the trachea and the surface properties of the wash were analysed by pulsating bubble (1 13) and by a modified *Wilhelmy* balance (Cahn Electrobalance) In addition, the cell content of the wash was determined using a Burker chamber

TABLE 1 *Surface Properties and Cell Content of Alveolar Wash at Various Intervals after Subcutaneous Injection of Paraquat 35 mg/kg*

Time after injection of paraquat (h)	No. of animals	Alveolar wash: Pulsating bubble amplitude ($cm\ H_2O$)	Wilhelmy balance hysteresis (dyn/cm)	Cell content (no/mm^3)
0 (controls)	4	0.41 ± 0.04	18 ± 6	128 ± 33
2	4	0.35 ± 0.14	12 ± 2	90 ± 25
4	4	0.33 ± 0.16	17 ± 6	93 ± 36
8	4	0.25 ± 0.04 }	13 ± 3	68 ± 15
12	4	0.21 ± 0.11 }	21 ± 3	83 ± 15
24	4	0.26 ± 0.16 }	7 ± 1	240 ± 102§

$p<0.02$ (combined groups versus controls)

§ $p<0.2$ (versus controls)

In tracings from pulsating bubble, the amplitude is defined as the difference between the pressure gradients across the bubble wall (ΔP) at maximal and minimal bubble size ($\Delta P_{max} - \Delta P_{min}$). In XY recordings from the Wilhelmy balance, the hysteresis between compression and expansion isotherms was measured at intermediate surface area.

As shown in Table 1 the bubble method revealed statistically significant reduction of pressure amplitude, reflecting surfactant derangement (13) 8–24 hours after paraquat injection. The Wilhelmy balance, on the other hand, revealed no significant abnormalities in the surface properties of the wash until 24 hours after paraquat injection. At this stage, however the cell content of the wash was somewhat increased.

Since we wanted to analyse the effect of paraquat on the alveolar lining layer at stage when a/ surfactant derangement can be established by physical methods and b/ considerable desquamation of alveolar epithelium has not yet occurred, the intervals 8 and 12 hours were chosen for our electron microscopic studies.

Ultrastructural Studies

The material used for electron microscopy consisted of four animals killed 8 hours after paraquat injection, and four animals killed 12 hours after paraquat injection. None of the animals had symptoms of respiratory discomfort and, throughout the series, the gross appearance and the histological appearance of the pulmonary parenchyma was normal.

The rats were anaesthetized with an overdose of intraperitoneal Nembutal and thereafter immediately tracheotomized. By means of cannula affixed in the trachea, the lungs were inflated under the endotracheal pressure of 10 cm of water. The lungs were then fixed according to one of the following methods, each method being applied to two animals at each interval.

Routine procedure The thorax was opened and the inflated lungs were submerged into 4 per cent glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2, room temperature) for 8–24 hours. This technique preserves the alveolar air-liquid interface during fixation (9). After fixation, small pieces (~ 1 × 1 × 2 mm) were cut from the margin of the lungs and rinsed for at least 3 hours in 0.1 M Na-cacodylate buffer (pH 7.2, 4 C). Postfixation was carried out with 1 per cent osmium tetroxide in Veronal buffer (pH 7.2, 4 C) for one hour thereafter the tissue blocks were rinsed in Veronal buffer (4 C) for 30 min. The blocks were then dehydrated in acetone (one hour at each concentration) and embedded in Vestopal W. Ultrathin sections were cut with an LKB Ultrotome, stained with uranyl acetate and lead citrate, and examined under a Siemens 1A electron microscope.

Ruthenium staining The routine technique was modified by the addition of ruthenium red (1 mg/ml) both to the glutaraldehyde and the osmium tetroxide solution (2). Otherwise the technique for preparation was identical.

RESULTS

Control animals had well expanded alveoli, covered with an acellular lining layer which filled dimples between bulging epithelial cells and smoothed out irregularities of the surface. In sections that had been fixed and stained according to the routine procedure, the lining layer was discontinuous. The hypophase of the layer was usually granular and



Fig. 1. Control rat: fixation according to the routine procedure. In this alveolus, the alveolar lining layer is well preserved. The hypophase contains numerous lattice figures, merging with the surface film. Lead citrate, uranyl acetate, × 31,500

contained numerous lattice figures at a spacing of 40–50 nm, some of which reached the air liquid interface to merge with the surface film (Fig. 1). In sections stained with ruthenium red, the alveolar lining layer was, as a rule, better preserved than in sections prepared according to the routine procedure. Furthermore, the electron density of the hypophase and the surface film was increased. However, the hypophase still appeared heterogeneous, granular areas alternating with lattice figures which merged with the surface film.

Animals killed 8 hours after injection of paraquat displayed some swelling and disintegration of mitochondria in granular pneumocytes. In addition, there was slight cytoplasmic swelling in membraneous pneumocytes and in alveolar capillary endothelium. The outer membranes of the degenerating alveolar epithelial cells, however, were not disrupted and there was no desquamation of epithelial cells. The alveolar lining layer had the same heterogeneous appearance as in control animals, i.e. there was a granular hypophase with numerous lattice figures, some of which reached the air-liquid interface (Fig. 2).



Fig. 2. Eight hours after paraquat injection, fixation with ruthenium red. The alveolar lining layer has the same heterogeneous appearance as in control animals (cf. Fig. 1). In the hypophase, an air bubble (*B*) is lined with an osmiophilic surface film. The cytoplasmic swelling of the membraneous pneumocyte indicated with *arrow* is an early effect of paraquat poisoning. Lead citrate, uranyl acetate, × 33 000.

The degenerative features in alveolar epithelium and alveolar capillary endothelium were more pronounced in animals killed 12 hours after paraquat injection. In fact, some membraneous pneumocytes were desquamated, leaving the basement membrane denuded. In such areas the alveolar lining layer was contaminated with cellular debris (Fig. 5). The lining layer thus appeared even more heterogeneous than in control animals, but lattice figures in contact with the surface film were still identified.

COMMENT

The functional integrity of the surface active substances of the alveolar lining layer probably depends upon a continuous excretion of phospholipids from the granular pneumocytes, the excretion being balanced by a re-

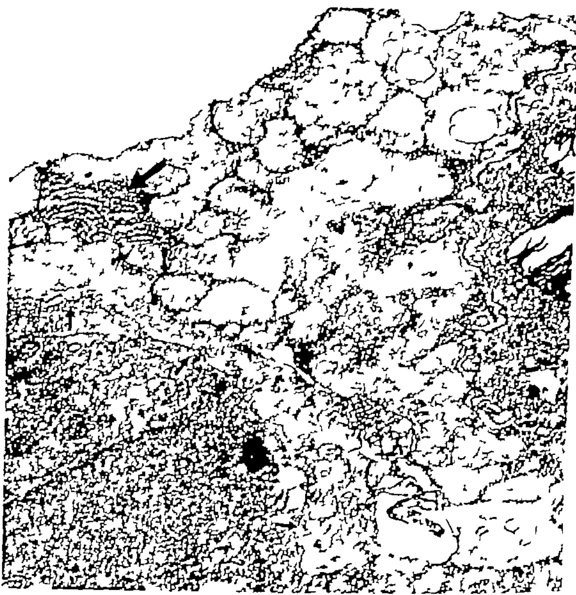


Fig 3 Alveolar wall of experimental animal twelve hours after paraquat injection. There is prominent, polypoid swelling and disintegration of membraneous pneumocytes fragments or extensions of such cells mix with ordinary constituents of the alveolar lining, e.g. the lattice figure indicated by *big arrow* *Right*, tip of granular pneumocyte *Small arrows* indicate basement membrane of alveolar epithelium. Ruthenium staining, lead citrate, uranyl acetate, × 36 000

moval of worn-out components of the surface film. Furthermore, the lining layer should be free from contamination with substances that might interfere with the physical properties of the surface film.

The combined evidence of the present and previous studies (6 8, 11 12) suggests that in paraquat poisoning there is derangement of the pulmonary surfactant system by way of at least two mechanisms. First the degeneration of alveolar epithelium that according to the present study begins already within 8–12 hours after injection of paraquat may impair the synthesis of surface active lecithin (6) Second, the alveolar lining layer is contaminated with cellular debris from degene-

rating epithelial cells this could explain the surfactant derangement observed 12 hours after paraquat injection. Perhaps a similar mechanism, e.g. contamination of the hypophase with plasma leaking out through the damaged alveolar wall, is responsible for the inactivation of surfactant that can be demonstrated by pulsating bubble as early as 8 hours after paraquat injection (Table 1) the present ultrastructural studies do not provide any conclusive evidence on this point. It might be added that paraquat is not surface active in itself and thus, it should not disturb pulmonary surfactant by competitive inhibition, nor does it inactivate lung surfactant *in vitro* (7)

In a frequently used theoretical model, the alveolar lining layer is regarded as a monomolecular film of phospholipids, overlying a hypophase which contains a reserve of phospholipid molecules. According to this concept, the surfactant molecules in the hypophase enter the air-liquid interface as the surface is expanded and join the hypophase again when the surface is compressed beyond equilibrium surface pressure. The time lag in the interchange of surfactant molecules between interface and hypophase will according to this theory explain the separation between the inflation and the deflation loops in pressure/volume recordings from intact lungs, as well as the hysteresis between compression and expansion isotherms in traditional recordings of surface tension versus surface area using the Wilhelmy balance. However the present as well as other ultrastructural studies of the alveolar lining layer (e.g. 3 4 5 14 15) suggest that this theoretical model is unduly simplified. As exampled in Figs. 1 and 2, numerous lattice figures take part in the air liquid interface at least in such areas, intermolecular forces operating between the various layers of the phospholipid complexes present immediately below the interface, probably influence the physical properties of the surface film as well as any postulated interchange of surfactant molecules between interface and hypophase

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CONGENITAL GENERALIZED LIPODYSTROPHY

Report on one Case with Special Reference to Postmortem Findings

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Berge, T., Brun, A., Hansing, B. & Kjellman, B. Congenital generalized lipodystrophy Report on one case, with special reference to postmortem findings. Acta path. microbiol. scand. Sect. A, 84 47–54 1976.

Generalized lipodystrophy (Berardinelli-Seip syndrome) was diagnosed in a boy at the age of 8½ months. Ten months later he died because of aspiration of food. Necropsy with special reference to the CNS revealed hypothalamic lesions, probably of a malformative or hamartomatous nature. The findings lend additional support to the view that hypothalamic dysfunction is responsible for the signs and symptoms of generalized lipodystrophy

Key words Lipodystrophy congenital generalized.

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Diencephalic lesions in infants can cause perplexing symptoms and growth disturbances.

A well-known syndrome of verified diencephalic origin (mostly due to glioma in the hypothalamic region) is the emaciation syndrome described by *Russell* (1951) Extreme paucity of subcutaneous fat is one of the cardinal signs of the syndrome. Initial acceleration of growth may occur large hands and feet have also been described (*Russell* 1951 *Gamstorp et al.* 1967) In cerebral gigantism which probably is due to a diencephalic lesion, gigantism and acromegaloid features dominate the clinical picture, but the subcutaneous fat is normal (*Sotos et al* 1964 *Kjellman* 1965)

In congenital generalized lipodystrophy the syndrome described by Berardinelli – Seip (B.-S.) the paucity of fat, the increased rate of growth and acromegaloid features are very pronounced (*Berardinelli* 1954 *Seip* 1959) In addition, there is muscular hypertrophy hypertrichosis, acanthosis nigricans, hepatosplenomegaly and disturbances in fat and carbohydrate metabolism. A few cases of abnormal pneumoencephalograms have been described and mental retardation is common (*Seip* 1971) According to the excellent review by *Seip* (1971) two features have mainly been suggested to explain the syndrome, viz. a primary disorder of the adipose tissue and, secondarily a disturbance of the hypothalamic—pituitary function.

So far the syndrome has not been encountered in Sweden, but 7 cases have been seen in Norway (*Seip* 1971 1975) As far as we know reports on post-mortem examinations of patients with the B.-S. syndrome are

not available. This paper describes a boy in whom a typical B.-S.-syndrome was diagnosed when he was 8 months old and who died from aspiration of food 10 months later. Interest was focused especially on the brain because of the assumed involvement of the diencephalon in cases of this nature.

CASE HISTORY

Y L., a boy born on November 24 1971 was the product of a nonconsanguineous marriage. The family history failed to add contributory data. Pregnancy and delivery in the 41st gestational week were normal. Birth weight 3070 gm., length 51 cm. He appeared dysmature, but the first few days were uneventful. When he was 9 days old, he had a urinary tract infection which responded favourably to chemotheraphy.

Because of his unusual appearance he was admitted to be re-examined at the age of 8½ months.

Psychomotor development was normal. His muscular strength, however, was exceptional and he could hang in one arm, the elbow being at right angle. He had an excessive appetite.



Fig. 1 The patient at the age of 8½ months.

Clinical Findings

Extreme paucity of subcutaneous fat, general muscular hypertrophy, hyperpigmented skin combined with hypertrichosis and large hands and feet were observed (Fig. 1). Length 75 cm, weight 9450 gm. The liver was palpable 8 cm below the costal arch. The spleen was not palpable.

Laboratory Findings

Routine analysis of the blood and urine revealed nothing remarkable. The following laboratory tests gave *abnormal* results: serum transaminases and total LDH were moderately increased, LDH electrophoresis disclosed an increase of fractions II and III, the serum cholesterol was 278 mg per 100 ml and triglycerides 1.4 mMol per litre. Lipid-electrophoresis disclosed a pattern corresponding to hyperlipidaemia of type II (Fredrickson). Generalised hyperaminoaciduria was in evidence and an oral glucose tolerance test showed a flat serum-glucose curve. Pertinent *normal* findings included skeletal age, fasting serum growth hormone at 2 nanograms per ml, unchanged after one hour sleep. Chromosome analysis was technically unsatisfactory.

At the age of 18 months, he was admitted to hospital because of massive aspiration of food. He succumbed in spite of intensive therapeutic efforts.

POSTMORTEM FINDINGS

General

Gross examination. The main features have been described under "Clinical Findings".

Weight 15 kg, length 96 cm. The weights of the organs are given in Table 1 together with the figures applying to normal children of the same length.

There was brown pigmentation of the skin around the neck and bilaterally in the axillary region. Subcutaneous f t could barely be demonstrated.

The lungs were partly atelectatic w th numerous small haemorrhages in the lower lobes. Large as well as small bronchi contained an bundance of aspirated food.

The left and the right ventricular walls of the hypertrophic heart, measured 25 and 4 mm, respectively.

The liver was substantially enlarged, almost reaching the iliac crest. The cut surface was of a pale yellowish-brown colour.

The spleen, tonsils and all the lymph-nodes were enlarged, the largest nodes measuring 2 cm in diameter.

Microscopic examination. Sections were taken from different parts of the organs given in Table 1 as well as from lymphnodes, tonsils, tongue, stri-

TABLE 1 *Organ Weights (Grams)*

Organ	Patient	Average (96 cm length which corresponds to 3–4 years of age)
Brain	1195	1165
Heart	150	84
Lung, right	285	145
Lung, left	170	124
Liver	1420	605
Spleen	110	74
Kidney right	120	124 right + left
Kidney left	123	
Adrenal, right	7.3	7.3 right + left
Adrenal left	9.0	
Thymus	85	19.5
Pancreas	90	33
Thyroid	9‡	

Average weights of organs according to body length. Data obtained from more than 30,000 paediatric autopsies. From Stowens, D. Pediatric Pathology. Second edition, p. 3. The Williams & Wilkins Co., Baltimore 1966.

‡ Corresponds to 8–10 years, (Abbott Abstracts)



Fig. 2. Pronounced subcutaneous lipodystrophy. Two eccrine sweat glands in the upper part of the figure at the border between dermis and subcutis. (×100. H+E.)

ated muscle, skin, prostate, testes, intestinal tract and bone marrow. The sections were stained with haematoxylin-eosin, van Gieson and Scarlet red. The pituitary was stained according to the Azan method for granules. There was generalized lipodystrophy (Fig. 2). The enlarged lymph-nodes had follicular structure with marked germinal centres. Sections of the enlarged liver showed severe fatty infiltration and fibrosis. The architecture was essentially preserved, but the portal triads were enlarged with fibrous septae which in some areas completely surrounded the acini. There was neither inflammatory reaction nor proliferation of small bile ducts (Fig. 3).

Histologically fat was demonstrated in the liver but not in lymph-nodes or spleen. Except for lipodystrophy histological investigation of other organs revealed no pathological changes.

Brain

Gross examination. The brain weighed 1195 gm. It showed mild hypoplasia of the suprasylvian portions of the frontal, central and parietal lobes and was evident also in the coronal sections. The posterior half of the callosal body was abnormally narrow and the internal capsule, cerebral peduncles and pyramidal tracts at the level of the inferior olives also appeared narrowed.

There was no widening of the ventricular system, including the third ventricle, and no gross change in the vicinity of the latter including the hypothalamus. The pineal body and the pituitary gland were of normal appearance.

The cerebellum was of normal size, shape and consistency.

The brain almost in its entirety was blocked for histological examination of whole brain paraffin sections covering all areas, particularly the hypothalamus. The sections were stained with haematoxylin-eosin, Nissl, Mahon for myelin and impregnated with silver for axons and neurofibrils, Sudan black B, PAS, long Ziehl Neelsen and Scarlet red. The last-mentioned was used for examination of selected small areas of the brain in frozen sections.

Microscopic examination. The thickness of the white matter of the frontal, central and parietal lobes was reduced, its myelin being of slightly reduced density and mild diffuse gliosis though focal scars were absent.

The perivascular spaces were slightly widened and partly filled with a loose connective tissue containing scattered macrophages. These had a granular cytoplasm that stained pale red with Scarlet red and was weakly PAS-positive, but negative in Sudan black B and long Ziehl Neelsen.



Fig 3 Fatty liver with portal fibrosis. No inflammation or ductulus proliferation. Several well preserved central veins. (×25 van Gieson.)

This staining reaction was found in many places around vessels in the white matter as well as in the cortex.

In the cortex of the frontal and central lobes, the pyramidal cells were slender with scanty cytoplasm, sometimes apparently bipolar showing simplified dendritic arborization. At this side, the lamination was indistinct and the cell arrangement was of a more columnar type. Neurons of the parieto-occipital lobes were triangular with wider cytoplasm the horizontal lamination was more prominent than that in frontal areas.

In the hypothalamus, multiple glial heterotopia were extending from the basal surface out into the *meninges where seemingly free heterotopias also was* found. Some of the heterotopic elements, e.g. those emerging from the mamillary bodies and the adjacent surface of the tuber cinereum, contained nerve cells which were streaming from the hypothalamus out into the pedunculated heterotopia. Other heterotopic structures consisted of glial cells and myelinated axons, but not arranged in any particular way (Figs. 4 and 5).

The walls of the ventral portions of the third ventricle showed a few small nodules composed of the subependymal type of glial cells and covered by ependyma which also formed rosettes in one of he nodules. This nodule was situated over the posterior hypothalamic nucleus (Figs. 6 and 7). The latter showed microvacuolization and fibrillar astrocytosis between its neurons (Figs. 8 and 9).

Other areas of the hypothalamus showed no conclusive alterations. The brain stem and cerebellum as well as the pituitary showed nothing remarkable apart from the above-mentioned narrowness of the cortic-spinal tracts. There were no focal lesions of anoxic type.

DISCUSSION

Judging from the clinical symptoms and signs as well as from the post-mortem findings, the diagnosis of the B.-S. syndrome is unquestionable in the present case. Signs of the disease were present already at birth, but had been misinterpreted as dysmaturity. Since *Seip* (1971) has recently surveyed the substance of our present knowledge about the B.-S. syndrome, we have confined ourselves mainly to the novel findings in the present case.



Fig. 4 Hypothalamic pedunculated hamartoma or glioneural heterotopia appearing as an inverted mushroom. (×30. H + E.)

Fig. 5 Part of Fig. 1. Stalk and body of hamartoma with large nerve cells. (×120. H + E.)

Fig. 6 Glioneural nodule attached to the wall of the inferior part of the IIIrd ventricle. (×42. H + E.)

Fig. 7 Part of Fig. 3. Glioneural nodule consisting of subependymal glia and ependymal rosettes. (×190. H + E.)



Fig 8 Posterior hypothalamic area with gliosis, microcystic degeneration and widening of perivascular spaces. (×100 H+E.)

Fig 9 The wall of the IIIrd ventricle showing gliosis and microcystic degeneration amidst hypothalamic neurons. (×250, H+E.)

In our case, the diagnosis was established at a relatively early stage,—the child was only 8 months old. This must be borne in mind when laboratory findings in the present case are compared with those obtained in other cases on record In the present case the hyperlipoproteinaemia was of type II (Frederickson's classification) whereas type V is the one mostly reported (*Seip* 1971)

In contrast to findings in most other cases, the skeletal development was not advanced according to the age of the child. The patient's weight ranged between the upper 2nd and 3rd standard deviation of normal. Length as well as weight corresponded to a chronological age of approximately three years. In Table 1 the weights of various organs are related to the normal means for length. It is seen that the organs were abnormally large, especially the pancreas and the thymus, which were 3 and 4 times the *normal* weight, respectively Only the brain weight was normal according to his length.

As observed in most other studies (*Seip* 1971) the *fasting serum-value of growth* hormones was not increased. It should be mentioned that, in the emaciation syndrome described by Russell, the concentration of the growth hormone is occasionally very high (*Häger* & *Thorell* 1973) In that syndrome involving an organic lesion in the diencephalon, the levels of serum lipoproteins may occasionally also be high (*Pierron et al.* 1967 *Pittagorliru et al.* 1969) Thus, the hyperlipoproteinaemia, regularly seen in the B.-S. syndrome, might also be interpreted as a manifestation of hypothalamic dysfunction.

Taking into consideration the available data on the B.-S. syndrome, Seip prefers the theory of a diencephalic origin over that of a primary disorder of the adipose tissue. This theory is strengthened by recent metabolic and physiological observations (*Mabry et al.* 1973 *Upton* & *Corbier* 1973)

The changes in the frontal and central cortex, the white matter and the corticospinal tracts were mainly of a hypoplastic nature. The cortical neurons seemed immature and the white matter was poorly developed with respect to myelinization and mass. This picture may occasionally be seen in perinatal trauma—usually in the form of hypoxia. Still, there was no history of symptoms or signs of a perinatal trauma. The distribution and type of cerebral changes were not equally compatible with a nutritional deficiency of which he showed no other evidence. The telencephalic findings, particularly the hypothalamic changes, might rather represent a malformation which in the cortex and white matter might manifest itself mainly in a retardation of maturation. Such changes may be found also in patients with moderate mental retardation.

The widening of the perivascular spaces and their accumulations of lipids may be reactions to the general lipid metabolic dis-

turbance. Judging from the staining reactions, the lipids appeared to be cholesterol esters. There were no signs of a progressive type of brain disorder and the hypoplasia of the corticospinal tract is most likely secondary to the telencephalic hypoplasia. This corroborates the diagnosis of a telencephalic lesion. *Seip* (1971) performed needle biopsy in one case, but found normal brain tissue without any abnormal deposition of lipids.

Pneumoencephalography has shown that the ventricular system, especially the third ventricle, may be dilated in some cases of generalized lipodystrophy (*Seip* 1959). Such dilation is common in cerebral gigantism (*Sotos et al.* 1964). The gross appearance of the ventricles was normal in the present case. Post-mortem estimation of the size of the ventricles of the brain is somewhat unreliable as the size of ventricles is smaller postmortally than during life (*Messert et al.* 1972). However a marked dilatation of the ventricles could be excluded.

The basal hypothalamic changes contributed clear evidence of damage to this area. The glial heterotopia or hamartomas, the subependymoglial nodules and the other changes in the posterior hypothalamic area justified the assumption of a hypothalamic lesion. These changes are unrelated to encephaloclasia and appear to be malformations.

A search of the literature failed to reveal any reports on the neuropathological aspects of such changes. The only exception was a report by *Witzgall* (1974) on a case of lipodystrophy acquired in adult age. In that case the hypothalamus showed less specific and prominent changes, mainly vascular hyalinosis, gliosis and neuronal regressive changes.

The significance of the hypothalamic lesions may be stressed since similar changes have been observed in e.g. pubertas praecox in which they are thought to produce symptoms either by hormone production by the neurons of the heterotopia or by mechanical effects on the hypothalamus (*Sheehan* 1968, *Wolman & Balmforth* 1963). In some cases of pubertas precox, however, other lesions have been found.

One mechanism of a hypothalamic dysfunction is a disturbance of the production of releasing factors from the diseased hypothalamus. The clinical symptoms may then be due to involvement of the pituitary gland or to direct effects on target organs by releasing factors which bypass the pituitary gland (*Besser & Mortimer* 1974).

Another possible mechanism of a hypothalamic dysfunction is a deficient regulation of the hypothalamus owing to damage to superior structures, a possibility which previously has been mentioned by *Nauta* (1963) and discussed by *Boström & Brun* (1971).

The exact pathophysiological role of the hypothalamic lesions in the present case remains to be explained. Deficient regulation of the hypothalamus caused by damage to superior structures (i.e. hypoplastic frontocentral lobes) cannot be excluded. Such mechanism might at least be partly responsible.

The findings lend support to the view based on clinical and laboratory findings, that generalized lipodystrophy is due to a hypothalamic dysfunction which may be primary or secondary.

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IMMUNOLOGICAL AND HISTOLOGICAL STUDIES OF TEMPORAL ARTERIES FROM PATIENTS WITH TEMPORAL ARTERITIS AND/OR POLYMYALGIA RHEUMATICA

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Waaler E., Tonder O & Milde, E. J Immunological and histological studies of temporal arteries from patients with temporal arteritis and/or polymyalgia rheumatica. Acta path. microbiol. scand. Sect. A 84 55–63 1976.

Biopsies from the temporal arteries of 62 out of 80 patients presenting the clinical picture of temporal arteritis and/or polymyalgia rheumatica showed morphologically active or healed arteritis. Fifty-five of these biopsies revealed anti-IgG activity as measured by the mixed agglutination test. In 21 of the 27 cases which could be completely studied, the anti-IgG activity was connected with the presence of IgA, either alone or together with IgG or IgM, or both, and complement. All of these 21 biopsies showed morphologically active granulomatous arteritis with signs of tissue destruction. In 6 biopsies, the active component appeared to be some type of Fc receptor in the tissue. Morphologically these biopsies showed either nongranulomatous mononuclear arteritis without definite necrosis or they represented various stages of healing arteritis with no or minor signs of tissue destruction. Weak anti-IgG activity was often found in a morphological type characterized by minimal inflammatory activity. These lesions are easily overlooked and the mixed agglutination test proved to be a good diagnostic tool in such cases. Arteries without anti-IgG activity showed no signs of active arteritis.

Key words Temporal arteritis polymyalgia rheumatica immunology histology

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In a preliminary report in 1968 (13) we described the presence of tissue-bound anti-γ-globulin activity in the temporal artery from one patient with granulomatous arteritis. This finding was later confirmed in biopsy material from 11 patients with this lesion (14 15). The activity was apparently connected with immunoglobulins in the tissue but the identification and classification of these immunoglobulins was incomplete

A larger material has now been collected in order to

1) classify the anti-Ig activity in the lesions,
2) study whether this activity is correlated to the histological patterns in the various stages of the disease and
3) examine the value of mixed agglutination as a diagnostic tool in these cases.

MATERIALS AND METHODS

Patients

With a view to diagnosis, temporal artery biopsies obtained from 184 patients were examined. According to the criteria defined by *Hamrin* (2) 80 of the patients suffered from temporal arteritis (TA) and/or polymyalgia rheumatica (PR). Forty five patients presented symptoms and signs of TA whereas 35 with PR had only general and myalgic complaints throughout the whole period of observation.

In 104 patients in whom TA or PR had been suspected at some time during their disease, the biopsies gave no indication of active or healed arteritis. The follow-up of these cases showed that the final diagnoses were malignant tumours, cardio-vascular disease of non-inflammatory nature various infectious diseases and connective tissue disorders. These patients represent our controls.

Biopsy Material

Biopsies from temporal arteries were frozen as soon as possible after surgery. In some hospitals, biopsies from patients were frozen in the operating room in Ringer solution and sent to the laboratory in the frozen state. The required number of cryostat sections were prepared for histological and immunological studies, up to 50–60 in series. Every fifth or tenth section was selected for histological investigation and stained with haematoxylin and eosin (HE). If enough material was available, formalin-fixed specimens were embedded in paraffin and sections were stained with HE and v. G eosin-elastin.

Sera and Serum Fractions

A pool of normal human serum was prepared from the serum of 10 healthy blood donors and stored at – 5 C in 0.5 ml aliquots. Normal rabbit serum was collected from animals which were to be immunized.

Antisera to sheep erythrocytes were produced by immunization of rabbits. The schedule consisted of weekly i tra enous injections for 6 to 8 weeks of 1 ml of a 5 per cent suspension of erythrocytes. The rabbits were bled when the IgG aggl tinins had reached a titre 1 1600 or bove. The agglutinating acti ty of the ntisera was determined by adding 0.. ml of a 1 per cent suspension of sheep erythrocytes to 0 2 ml of twofold dilutions of the sera. Phosphate buffered saline pH 7.2 (PBS) served as diluent. The titre of the serum was given as the reciprocal of the highest dil tion which gave definite aggl tination. One agglutinating unit is defined as the amount of the highest dilution of antiserum which agglutinates an equal amount of a 1 per cent suspension of sheep erythrocytes.

Monospecific antisera to human IgG IgM and IgA were prepared as described earlier by immunization of rabbits, using their own erythrocytes agglutinated by IgG IgM or IgA antibodies, respectively (4 11). Rabbit antiserum to human Clq was provided by Dr S Thunold Department of Pathology The Gade Institute, Bergen, Norway It was produced as earlier described (10). Commercial rabbit antisera to human IgA, IgG, IgM, IgE and IgD and to C3 were purchased from Behringwerke AG Marburg Lahn, West-Germany For inacti ation of complement, the sera were heated to 56 C for 20 min (inactivated sera). Treatment of sera with 2-mercapto-ethanol (ME) was performed as previously described (6)

Human IgG (Fraction II 16.5 per cent solution) was purchased from AB Kabi, Stockholm, Sweden. Rabbit IgG (lyophilized Fraction II) was obtained from Pentex Inc., Kankakee Ill., USA.

Denatured IgG was prepared by heating a 4 per cent sol tion in PBS (w/v) for 10 min at 63 C.

Isolated IgG was digested by pepsin using the method described by *Nisonoff* (7)

Various Reagents

Pepsin (2 × crystallized) was purchased from Sigma Chemical Co., St. Louis, Mo., USA. Mercapto-ethanol iodo-acetamide, formaldehyde and acetone were used as standard reagents.

Demonstration of Anti-IgG Activity in Tissue Sections

Precise technical details of mixed agglutination with tissue sections have been described elsewhere (6). Indicator cells (EA) were prepared in the same way as those used for demonstration of rheumatoid factor in tissue sections (6). Sheep erythrocytes (E) were sensitized by rious agglutinating and subagglutinating amounts of rabbit IgG antibodies to E (A). Larger amounts than 4 units could not be used because EA formed too large clumps.

Sensitization was performed by mixing equal volumes of a 1 per cent suspension of sheep erythrocytes and diluted antiserum. The mixture as left at room temperature for 10 minutes. After washing, the erythrocytes were made up to 1 per cent suspension in PBS containing per cent NaCl.

Air dried cryostat sections of frozen tissue on large coverslips were incubated at room temperature in moist chambers together with the indicator cells applied, using microculture slides with a single concavity allowing EA to settle on the coverslip. After 30 min, the slides were turned and left for detachment of EA from the glass and nonreacti e tissue. Haemadsorption was recorded mi-



Fig 1 Frozen sections of temporal artery biopsy 5–6 months after onset of symptoms. a: Diffuse granulomatous inflammation with fibrinoid necrosis and infiltration of macrophages and lymphocytes, mainly in adventitia and media, H.E. × 45 b Neighbouring section of a, mixed agglutination test. The sensitized cells adhere to the tissue 3+ reaction, × 45.

Fig 2 Frozen sections of temporal artery biopsy 11 weeks after onset of the disease. a 3+ mixed agglutination test unstained section is seen outside the adhering sensitized cells, × 75. b Same block; mixed agglutination test after inhibition with antiserum to IgA, IgG and IgM. No adherence of sensitized cells the unstained section is visualized.

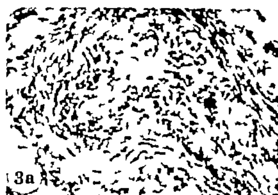
croscopically when the glass around the tissue was free of erythrocytes. In strongly positiv reactions (3+) the tissue was completely covered by erythrocytes (Figs. 1 b and 2 a) In moderately (+) and weakly (1+) positiv reactions, partial covering of the tissue was observed (Figs. 3 b and c) If no erythrocytes were attached to the tissue the reaction was termed negativ (–)

The tissue activ ty was graded as strongly re active when positive reactions were obtained w th EA sensitized by subagglutinating amounts of A ( ); or / unit) If positiv reactions were obtained only with EA sensitized by 4 and 2 units of A, the tissue was graded weakly reactive and medium reactiv if the reactions were obtained with one unit.

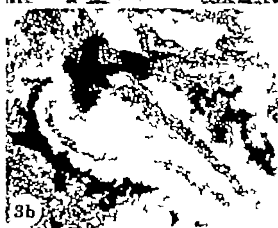
Pret atment of Tissue Sections

For some experiments the sections were exposed to 4 per cent formaldehyde in PBS ether acetone or 96 per cent ethanol before testing with EA. Coverslips with the sections were submerged for 2 min in beakers containing the reagent.

In inhibition tests, the sections were first incubated w th the serum fractions or the various antisera to serum proteins described above. Tissue sections were screened using sera diluted 1 in 2 or IgG in 4 per cent solution and, if the reaction between tissue sections and EA were inhibited (Fig. 2 b) the inhibiting agent was further tested in twofold dil tions on the sections. Most of the tissues were also incubated with the various anti-


3a


3c


3b


3d


4


5

sera combined. The coverslips were placed in moist chambers and kept at room temperature for 30 min. Thereafter excess of the reagents was removed and the sections were washed by submerging the coverslips vertically into beakers with buffered saline for 10 min.

For treatment with ME, one drop of a 0.2 M solution was placed on each section. After incubation for 30 min at room temperature, one drop of 0.2 M iodo-acetamide was added and the sections were left for another 30 min. Thereafter the sections were washed in saline.

The sections treated in these various ways were then tested in mixed agglutination.

RESULTS

Clinical Picture in Relation to Histological and Immunological Findings

Table 1 summarizes the results obtained in our series including 104 control cases and 80 patients with TA and PR. In all 104 biopsies from control cases, the temporal arteries were without signs of inflammation or sequelae of the latter. It was only in 2 cases that sections of such arteries showed weak anti-IgG activity.

All patients presenting the clinical picture TA had arteritis or sequelae of the latter. Among those with PR, only 17 patients (50 per cent) had active arteritis or older lesions, while arteries in 18 patients altogether were without any signs of inflammation. The arteries from all patients in the latter group showed no anti-IgG activity, whereas such activity in the arteries was observed in 40 of the 45 patients with TA. The results were always reproducible in repeat tests, except for some variations within the group which showed the weakest reactions.

Histological Classification

In order to compare the histological and immunological findings it is necessary to have a simple histological classification and to give a survey of the staging and of the transitional or borderline pictures. The following 4 categories proved to be useful:

a) Granulomatous inflammation, diffuse and/or nodular
b) Non-granulomatous mononuclear arteritis
c) Regenerative lesions and scars.
d) Minimal non-characteristic lesions.

Granulomatous arteritis. This is the common histological finding in the active stage of the disease. There is a granulomatous inflammation with infiltration of macrophages and giant cells mainly in the media. Fibrinoid necrosis is seen and small "rheumatoid granulomas" may occur. The intima is markedly thickened with cellular infiltration mainly in the outer zone. In the adventitia, lymphocytes dominate.

Non-granulomatous mononuclear arteritis. This category comprises lesions involving diffuse infiltration of lymphocytes, mainly in the media and adventitia (Fig. 4). The arteries are markedly thickened and proliferation of fibroblasts is varying. There are few or no macrophages and necrosis does not occur.

Regenerative lesions and scars. This category comprises cases of advanced scar lesions and late regenerative changes (Fig. 3 a and d). There is lymphocyte infiltration, marked to scanty or none in the completely burned-out

Fig. 3. Sections from temporal artery biopsy 10 days after myalgic relapse; temporal arteritis 8 years earlier. a: Minor inflammatory lesion, reactivation? H.E. × 70. b: Neighbouring section to "a" shows a 1+ mixed agglutination test. The sensitized cells (black) can be seen against the background of the unstained section, × 70. c: Same section as b showing the individually adhering sensitized cells, × 370. d: Paraffin embedded material from the same artery. The section shows a scar lesion with fibrosis and infiltrating lymphocytes.

Fig. 4. Frozen section of temporal artery biopsy 9 weeks after onset of symptoms. The section illustrates the "non-granulomatous mononuclear arteritis". Thickening of the intima, moderate diffuse lymphocytic infiltration, slight proliferation of fibroblasts, no macrophages and no fibrinoid necrosis, H.E. × 70.

Fig. 5. Frozen section of temporal artery biopsy 9 weeks after onset of symptoms. The section illustrates the minimal non-characteristic lesion. Intima thickened and small focus in the media with lymphocytic infiltration. H.E. × 175.

TABLE 1 *Temporal Arteritis (TA) and Polymyalgia Rheumatica (PR) Distribution of Number of Individuals According to Clinical Diagnoses Histological Findings and Anti-IgG Activity in Sections of Temporal Arteries from 80 Patients and 104 Control Cases*

Clinical diagnoses	Findings in sections of temporal arteries				Total
	Histology		Anti-IgG activity		
	Inflammation*	No inflammation	Present	Absent	
TA	45	0	40	5	45
PR	17	18	15	20	35
Control cases	0	104	2§	102	104

* Including active inflammation and sequelae.

§ One patient with rheumatoid arthritis and one with carcinoma of the ovary

TABLE 2. *Distribution of Number of Patients According to Histological Category and Strength of Anti-IgG Activity in Tissue Biopsies from Temporal Arteries*

Histological category	Anti IgG activity					Total
	Strong	Medium	Weak	Not graded	Absent	
Granulomatous inflammation	19	14	4	1	1	39
Non-granulomatous mononuclear arteritis	1	2	1	1	1	6
Regenerative lesions, scars	2	2	3	0	4	11
Minimal lesions	0	0	4	0	2	6
No inflammation	0	0	1	0	17	18
T tal	22	18	13	2	25	80

cases, and no necrosis. Transitional stages to the active inflammatory lesions may occur The age of the proliferating connective tissue determined the classification.

Minimal non-characteristic lesion. There is a more or less marked thickening of the intima and a slight infiltration of lymphocytes in all three layers of the artery but the cellular infiltration may be scanty and limited to the adventitia (Fig. 5) Examples of this category may be found outside or close to granulomatous lesions. However the minimal lesion may be the only finding and, if so it is easily overlooked or misinterpreted.

Comparison of Anti IgG Activity and Histological Category

The various histological categories were compared with the strength of anti-IgG activity as measured by mixed agglutination (Table 2) Strong anti IgG activity was found, often in connection with granulomatous arteritis (approx. 50 per cent) but also in non-characteristic and old regenerative lesions and scars. The cases with minimal arteritis showed weak reactions.

Biopsies from altogether 25 patients showed no anti-IgG activity wheras 17 of these only were "negative" histologically Absence of anti IgG activity was found in 4 old scar lesions and in 4 presenting active inflammation. In the latter cases, the inflammatory activity varied considerably in the different segments and our histological controls indicate that the sections used for mixed agglutination had not been representative.

The table shows one "false" positive mixed agglutination with an artery without signs of

TABLE 3 *Distribution of Number of Patients According to Class of Anti-IgG in Tissue Biopsies from Temporal Arteries*

Class of anti-IgG	Number
IgA	11
IgG	1
IgM	1
IgA + IgG	5
IgA + IgM	3
IgA + IgG + IgM	2
Other reactors	6
Doubtful results	4
Total	31

Anti-IgG of complement nature (C1q) was present in all tissues exhibiting anti-IgG of immunoglobulin nature.

inflammation. The results of the mixed agglutination test varied in this biopsy where some sections were positive and some negative. This latter case is not included in the following presentation.

Classification of the anti-IgG activity. The results presented above indicated a significant relationship between histology and anti IgG activity. Further experiments were then designed to identify the components which were responsible for this activity. Most of the following results were obtained in inhibition experiments. Tissue sections were incubated with human and rabbit IgG and the antisera to human immunoglobulins and complement before the test with EA. The weakest sensitized EA which gave a 3+ or 2+ reaction with untreated sections was used in these experiments. Most of the positively reacting arteries showed no reaction with the EA after incubation with the denatured IgG fractions, indicating a rheumatoid factor like anti-IgG activity.

The data presented in Table 3 are based on the results obtained with the antisera. If each of 2 or more of the antisera gave partial inhibition, giving complete inhibition if combined, this was interpreted to indicate that the anti-IgG activity was connected with 2 or more components. F inst. if anti-IgA, anti-IgM and anti-C1q each gave partial inhibition though complete inhibition if combined, the anti-IgG activity was classified as being connected with IgA, IgM and complement.

Biopsies of altogether 21 arteries revealed anti IgG activity of Ig type and complement (mainly C1q). IgA being the dominant component, either alone or together with IgG or IgM, or both. In biopsies from 6 patients, "other reactors" appeared to be responsible for the positive, mixed agglutination reactions. These reactions were inhibited by all antisera in low dilutions and 4 were completely inhibited by human and rabbit IgG. Apparently tissue receptors presenting a broad reactivity were responsible for these reactions. Using biopsies from 4 patients, the inhibition studies gave varying results but the material was insufficient for further studies.

The anti-IgG reactions were in all cases

TABLE 4 *Distribution of Number of Patients According to Histological Category and Type of Anti-IgG Activity in Tissue Biopsies from Temporal Arteries*

Histological category	Type of anti-IgG activity			
	Ig + C	Other reactors	Doubtful results	Total
Granulomatous inflammation	21	1	4	26
Non-granulomatous	0	1	0	1
Regenerative lesions, scars	0	4	0	4
Minimal lesions	0	0	0	0
Total	21	6	4	31

C = complement.

dependent on an intact Fc portion of the IgG molecule in the EA indicator system as no reactions were obtained if A were substituted by the F(ab)$_2$ fragment. Sections fixed in 4 per cent neutral formaldehyde, ethanol or acetone showed no activity whereas ether treatment had no influence upon the reactions. Sections treated with ME showed similar or stronger activity as compared with untreated sections.

Ig class and histological category. Tissue-bound immunoglobulins and complement were found in 21 biopsies all presenting granulomatous arteritis (Table 4). The class or classes of tissue-bound immunoglobulins did not appear to have any relation to the histological picture.

In 4 of the 6 biopsies which showed other reactions, the histological picture was dominated by proliferative changes and lymphocytes, one non-granulomatous arteritis, 2 in the regenerative-scar category and one granulomatous arteritis. The latter biopsy showed great variations in the different segments, many sections were dominated by regeneration. Two biopsies of the regenerative-scar category were almost "burned-out" lesions with scattered lymphocytes.

DISCUSSION

The present results substantially support our
[illegible] ies in biop-
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completely
nti-IgG ac
globulins or
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gG activity
gether with
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e as inhibi-
rabbit IgG
urification,
o activities
This accords well with the fact that C1q reacts with aggregated IgG and antigen-antibody complexes.

That immunoglobulins and complement are present in these arteries accords well with results obtained in other studies of temporal arteritis (5, 8), of small-vessel vasculitis (9), mixed cryoglobulinaemia (9), systemic lupus erythematosus (3) and rheumatoid arthritis (1). Circulating immune-complexes have been implied in the pathogenesis of the vascular lesions in each of these diseases. At the present stage of investigation we cannot say whether the immunoglobulins observed in the tissue result from the passive deposition of immune-complexes where rheumatoid factor is already bound to the complexes or from the combination of specific antibodies with some unknown antigens in the arterial wall. More extensive studies are necessary to identify fully the mechanism behind the deposition of immunoglobulins and complement in the temporal arteries in these diseases.

However, on the basis of our results we can to a certain extent elucidate the three problems stated in the introduction.

1) The anti IgG activities demonstrable in granulomatous arteritis are most often of Ig nature, IgA being dominating. In this respect, the anti IgG activity is different from the activity which is characteristic of adult rheumatoid tissue, i.e. rheumatoid factors belonging to the IgM class. The presence of IgA anti-IgG factors in the tissue together with complement strongly indicates that antigen/antibody complexes or otherwise denatured immunoglobulins are present in the tissue. That is to say that temporal arteritis *is a disease* which shows many similarities with connective tissue diseases. We are unable to determine whether the immunoglobulins and complement play any active role in the pathogenesis of this disease.

2) Our studies indicate that there is a definite relationship between the clinical-histological stages and the presence of anti-IgG activity in the tissue lesions. In active granulomatous arteritis involving tissue destruction, immunoglobulins and complement are bound

in the diseased arteries, whereas the old, healed scar stages are characterized by complete absence of immunological activity

There is further a group of patients with non-necrotic proliferative inflammation in the arteries where reactors other than immunoglobulins and complement are acting In 4 out of 6 cases in this group B-lymphocytes or monocytes known to carry membrane bound Fc receptors (12) may be the agents which bind EA.

3) In our hands, the mixed agglutination test has proved to be an efficient and guiding tool in the routine diagnosis of TA. The demonstration of weak anti-Ig activity in biopsy specimens presenting minor non-characteristic reactive changes regularly led to a re-evaluation of the biopsies. The weak activity alone cannot be regarded as a diagnostic proof but may support the diagnosis of arteritis in cases where suspicious, but not definitely diagnostic, histological changes are present. Thus, some of the cases in our series registered as minimal, non-characteristic, chronic arteritis would probably have passed unnoticed without the guiding help of the mixed agglutination test. The fact that identical changes occurred in connection with granulomatous arteritis indicates that these minimal inflammatory lesions are part of the morphological picture in these conditions.

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ULCER FORMATION AND HISTOCHEMICAL CHANGES IN RAT-STOMACH MUCOSA INDUCED BY ACETYLSALICYLIC ACID

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Jørgensen, T G Ulcer formation and histochemical changes in rat-stomach mucosa induced by acetylsalicylic acid. Acta path. microbiol. scand. Sect. A, 84 64–72, 1976.

Numerous papers have emphasized the damaging effect of salicylic acid compounds on the stomach mucosa. The investigations here reported aimed at examining the effect on gastric and duodenal morphology of a moderate dose of aspirin. Oxidative enzymes were evaluated histochemically To rats in groups of 16, aspirin tablets, placebo tablets, or the same with additional 1 cc of 0 1 N HCl, were administered twice a day for 6 weeks. Approximately 160 mg/kg/24 hrs were ingested Microscopy revealed severe hyperaemia along with focal gastritis in the aspirin treated animals. Chronic gastric ulcers were observed in 18 rats in the aspirin treated groups, particularly in the group where HCl was added. Ulcerations in the area of the pyloricgland occurred in the latter group only A reduction in mucopolysaccharides was demonstrated and involved the acid as well as the neutral ones. NADH-reductase and cytochrome oxidase were inhibited not only in the surface cells but also in those in the deeper layers. The reduction in oxidative enzymes in otherwise undamaged areas suggests an interference with the cellular metabolism, probably processes in the respiratory chain which might lead to reduction in energy-rich phosphate bonds.

Key words Gastric ulcer acetylsalicylic acid histochemistry

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The damaging effect of salicylic acid and its derivatives on the stomach mucosa has been established in numerous papers.

Acetylsalicylic acid is of significance in that it provokes exacerbations of ulcer symptoms (24) and, in the aethiology of acute upper gastro-intestinal bleeding provokes superficial erosions (10) Further clinical and epidemiological evidence has been presented suggesting that aspirin is the aethiological factor in some cases of gastric and duodenal ulcer (5 13) The different investigations have focused on different pathogenetic factors. Considerable research has been carried out with a view to establishing the effect on mucous secretion (23) the increased exfoliation of gastric epithelial cells

(7) back-diffusion of hydrogen-ions (9) and changes in acid secretion (14). The processes mentioned are all dependent on an energy supply derived from oxidative processes in the mucous membrane. The following study was carried out with a view to examining the effect of a moderate dose of acetylsalicylic acid on gastric and duodenal morphology and on oxidative enzymes of the mucosal membrane.

MATERIAL AND METHODS

Sixty-four Wistar rats weighing around 250 g were divided in 4 groups, each containing 16 rats: 8 males and 8 females. The rats were kept on a standard laboratory diet and had free access to water. Tablets containing 20 mg of acetylsalicylic acid or 20 mg of placebo (lactose + amylum solani) were manufactured according to the usual procedure (Pharmacopea Nordica). A piston with a diameter of 2 mm was used for stamping the tablets the height of which was 2½ mm. Twice a day during 6 weeks, one tablet was administered. The tablet was easily swallowed by the rats if they were held upright and the tablet, by means of a forceps, was placed behind the tongue. The amount of acetylsalicylic acid or placebo administered was 40 mg a day corresponding to a dose of around 160 mg/kg/24 hrs. In addition, animals in 2 of the experimental groups had 1.0 cc of 0.1 N HCl administered by gavage just after ingestion of the tablet. Table 1 summarizes the treatment of rats in the different experimental groups.

At the end of the six weeks period, the rats were sacrificed by a blow to the head. The abdominal cavity was rapidly entered, the stomach and the proximal duodenum were removed and opened along the greater curvature, the non-glandular portion being excised. The stomach-duodenum preparation was pinned on a pre-cooled cork. Large food particles were removed by a soft brush and photographs were taken using standard conditions, i.e. fixed light and distance. (Kodachrome II colour diapositive film). The specimen was cut into halves along the smaller curvature. One half was immediately frozen in a mixture of dry ice and acetone for histochemical reactions and the other half was fixed in 10 per cent formaldehyde-Ca and embedded in paraffin wax. Stepwise sections were cut at 5 µ from each half.

TABLE 1. *Aspirin Effects on Gastric and Duodenal Mucosal Membranes in Rats*

Experimental groups — compounds administered

Group	Aspirin 40 mg/24 hrs	Placebo 40 mg/24 hrs	HCl 2.0 cc 0.1 N
A. (16)	+	—	—
B. (16)	+	—	+
C. (16)	—	+	—
D. (16)	—	+	+

Acetylsalicylic acid and placebo tablets manufactured according to Pharmacopea Nordica.

( ): number of rats in each group at start of experiment: 8 ♂ 8 ♀

Stains and Histochemical Reactions

The following staining reactions were used:

Haematoxylin-eosin and van-Gieson staining, the latter in combination with the Alcian-blue staining method for acid polysaccharides using Alcian-blue 8 GX at pH 2.6 (32). The gastric mucosa was stained by the method of *Zimmermann* (36) according to the modification described by *Marks & Drysdale* (19) replacing the mucicarmin by the PAS-stain of *McManus* (22).

ATP phosphohydrolase (ATP-ase) was demonstrated by the calcium method according to *Padykula & Herman* (28). Sections were cut on a freezing microtome and incubated for 30 minutes at 37° in a medium at pH 9.4 containing ATP as substrate. The tissue sections were counterstained with haematoxylin. It was not attempted to separate the non-specific alcaline phosphatase activity. Control-sections were incubated in medium without ATP. If ATP was present, sites of ATP phosphohydrolase would appear as black deposits (cobalt sulphide).

NADH reductase (NADH-diaphorase) was demonstrated by incubating cryostatic sections for 30 minutes at 37° in a medium at pH 7.2 containing cobalt, NADH (substrate) and 2.5 diphenyl tetrazolium bromide as H- and e-acceptor (31). Sites of diaphorase activity appeared as black deposits of cobalt formazan. Control-sections were incubated in the same medium without NADH.

Cytochrome oxidase was demonstrated by a modification of the amine coupler method (26) using a reaction medium containing phosphate buffer (pH 7.4), α-napthol, 4-amino-1 N-N-dimethylnaphtylamine, cytochrome c and catalase. Frozen sections were incubated at 37° for 30 minutes. Sites of cytochrome oxidase activity appeared as brownish-black granules. With a view to controlling the reaction, sections were incubated in a medium to which KCN (10^{-3} M) was added. No pigment deposits occurred in any of the control sections.

The histochemical reactions were evaluated semiquantitatively graduated: +, ++, +++. The









Fig 1 (upper left) Leucocytes infiltrating the mucosa in the are of the corpus gland Aspirin treated rat. Haematoxylin-eosin. × 140

Fig 2 (upper right) Large erosion in the area of the corpus glands. The rat had ngested aspirin with HCl added. Haematoxylin-eosin × 140

Fig 3 (lower left) *Aspirin tablet particle (arrow) buried in the mucosa and surrounded by necrotic* cells. Haematoxylin-eosin. × 140

Fig 4 (lower right) Ulceration of the rea of the corpus glands. The bottom of the ulcer crater is covered by a loose connective tissue. Only a few leucocytes are present and fibrous l ne rly absent. Van Gieson-Alcian. × 14

interpretation of the histochemical reactions was carried out following blind study of coded sections.

Statistics

The exact hypergeometric test was used.

RESULTS

Four rats died during the experiment. They all belonged to the groups in which acid was administered and they all died during the first week, probably from aspiration. In one case, autopsi showed severe changes in the stomach mucosa. Large areas of confluent erosions and two dark, necrotic points were found after 4 days of aspirin + HCl.

Macroscopical estimation of the degree of damage on the basis of the photos

Comparison of group A (acetylsalicylic acid) and group C (placebo) demonstrated only minor changes, i.e. the colour of the mucous membranes in group A was more brightly red especially in the area of the pylorus glands and a few slightly bleeding points could be seen, especially in the area of the corpus glands.

No macroscopically detectable lesions could be seen in group D (placebo + HCl) or in group C. In group B (acetylsalicylic acid + HCl) the number of erosions exceeded that in group A. As in group A, the area of the pylorus glands appeared somewhat hyperaemic. In group B, an ulcer 1.5 mm in diameter was seen in two of the preparations. These ulcers were located at high sites in the area of the corpus glands.

No differences between male and female *rats could be demonstrated in any of the groups.*

Microscopic findings, general description

A round cell infiltration in the submucosal layer was seen in control groups as well as in the groups receiving acetylsalicylic acid. The predominant type of cell was the plasma cell. In the aspirin treated rats, the number of infiltrating cells was far greater than that in controls, and the infiltration occurred in the mucosal membrane itself (Fig. 1). Superficial erosions were widespread in the aspirin treated animals, particularly in the group which had HCl in addition to aspirin (Fig 2). Erosions were seen even in the placebo group, but the number and extension was limited. The aspirin induced erosions were located mainly in the area of the corpus glands. No erosions occurred in the duodenal mucosal membrane. In general, the erosions reached the depth at the level of the isthmus part of the corpus glands, but deeper erosions occurred as well as partly healed erosions, identified through the occurrence of primitive glands.

Congestion of mucosal capillaries was seen only in the aspirin treated rats, often with concomitant mucosal and submucosal oedema and round cell infiltration. In the *aspirin + HCl* group especially extravasation of erythrocytes occurred if the capillaries were involved by the damage.

Eosinophilic corpuscles were frequently seen to be embedded between the mucosal folds (Fig 3). By means of their eosinophilic staining characteristics these corpuscles could be identified as pieces *of ingested* tablets. In the acetylsalicylic acid treated groups, the contact with these particles of tablets appeared to produce a severe reaction of oedema and capillary *erosion*

Lesions were observed in the sections from 18 *rats* these lesions penetrated the muscularis mucosa and resembled chronic gastric ulcers with fibroblastic *proliferation* and col-

TABLE 2. *Chronic Gastric Ulcers in Rats after 6 Weeks of Aspirin Ingestion (160 mg/kg/24 hrs.)*

Group		Number of ulcers: Area of corpus glands	Number of ulcers: Area of pyloric glands
A. aspirin	(16)	5	0
B aspirin + HCl	(13)	8	8
C. placebo	(16)	0	0
D placebo + HCl	(15)	0	0

Numbers in brackets number of rats at the end of experiment (4 died during the experiment)

2 mucosal membranes demonstrated 2 and 3 ulcers, respectively

A + B/C + D $P < 0.01$ A/C: $P = 0.04$ B/D $P < 0.01$ B/A $P = 0.025$

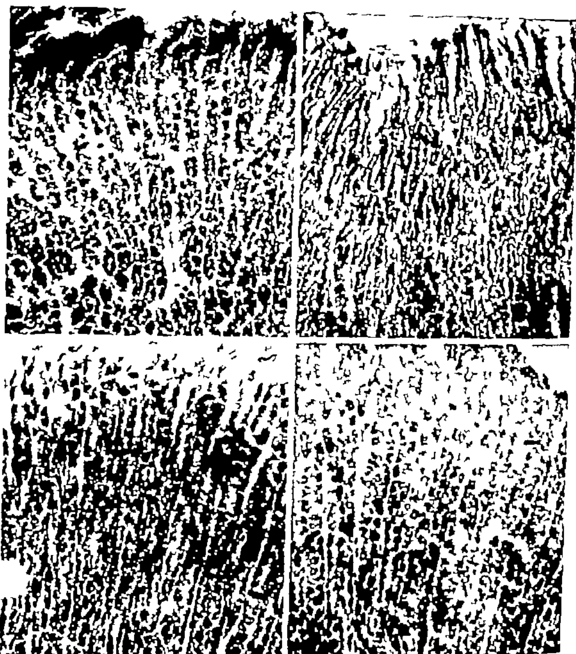


Fig 5 (upper left) The NADH-reductase reaction in the area of the corpus gland in placebo treated rat. The reaction is strongly positive in the surface- and isthmus mucous cells and in the cytoplasm of the parietal cells. × 280

Fig 6 (upper right) NADH-reductase reaction following aspirin + HCl ingestion. A reduction of the reaction in the surface- and isthmus mucous cells is seen. × 280

Fig 7 (lower left) Cytochrome oxidase reaction in the area of the corpus gland in placebo treated rat. The enzyme is present especially in the cytoplasm of the parietal cells. A fairly weak reaction takes place in mucous- and chief cells. × 280

Fig 8 (lower right) Cytochrome oxidase reaction following aspirin + HCl ingestion. Areas of strongly inhibited reaction are seen. Especially the reaction in the parietal cells is inhibited. × 280

TABLE 3 *NADH Reductase and Cytochrome Oxidase Reaction in Rat Stomach and Duodenal Mucosa Following Placebo or Aspirin Ingestion for 6 Weeks*

NADH-reductase	Corpus area: Surface epithelial cells	Corpus area: Foveolar and neck cells	Corpus area: Parietal cells	Corpus area: Chief cells	Pyloric area: Surface epithelial cells	Pyloric area: Foveolar and pyloric gland cells	Duodenum: Epithelial cells
Placebo or placebo + HCl	+ +	+ +	+ + +	+ +	+ +	+	+ + +
						+	(+ +)
Aspirin	+	+	+ + +	+	+		
Aspirin + HCl	+	+	+ +	+	+	+	(+ +)
Cytochrome oxidase							
Placebo or placebo + HCl	+ +	+	+ + +	+	+ +	+	+ +
Aspirin	+	+	+ + +	+	+	(+)	+ +
Aspirin + HCl	+	+	+ +	+	+	(+)	+ +

Parentheses indicate inconstant findings.

lagen at the base (Fig. 4). Twenty-one ulcers of this type were seen (Table 2). The size of the ulcers ranged from 0.1–1.5 mm in diameter.

Any difference between ulcer frequency in male and female rats was not found. Some of the ulcers demonstrated a healing tendency, primitive mucosal glands growing in from the edge of the mucosal defect.

Results of the Histochemical Studies

The histochemical reactions in the placebo group did not differ from those in the group of non-treated rats.

Acid mucopolysaccharides assessed as Alcian-positive substance occurred predominantly in cells in the neck of the corpus gland, and in the deep foveolar cells in the area of the pyloric glands (+ + +). Following aspirin or aspirin + HCl ingestion, a significant reduction occurred in both of the gastric mucosal areas in all sections. The mucin secreting cells all stained strongly with the PAS-reaction (+ + +). Administration of aspirin or aspirin + HCl led to a slightly weaker reaction (+ +) in the surface epithelium and in foveolar cells in the area of the corpus and the pyloric glands, whereas the reaction was further reduced in cells in the neck of the glands (+).

A pronounced ATP-ase reaction occurred particularly in the surface epithelium of the area of the corpus- and pylorus glands as well as in duodenum. Furthermore the parietal cells demonstrated a strong positive reaction. Neither aspirin nor aspirin + HCl had any demonstrable effect on the distribution or intensity of the reaction.

The reaction for NADH reductase and cytochrome oxidase appear from Table 3. Ingestion of aspirin or aspirin + HCl led to an inhibition of NADH reductase reaction, especially in the superficial epithelial layers (Fig. 5, 6). This finding was present in all the sections. Large areas with reduced reaction were found throughout the stomach mucosa in the sections from aspirin + HCl treated rats. This phenomenon was observed in all but one of the rats in that group. The inhibition of the reaction in the duodenal epithelium was not a constant finding.

Following aspirin or aspirin + HCl ingestion, an inhibition of the cytochrome oxidase reaction in the surface mucous cells occurred in all but three sections. Reaction of the cells of the pyloric gland was inhibited in half of the sections, whereas the reaction in parietal cells was found to be inhibited in

all but two sections from aspirin + HCl treated rats. Furthermore, the latter group exibited sporadical areas without reaction especially in the corpus mucosa (Fig 8)

Both of the enzyme reactions were seen to be inhibited in the mucosal cells limiting erosions and ulcerations

DISCUSSION

Except for the two ulcers, the macroscopic changes observed were similar to the erosive changes seen in acute experiments (8, 37) Following aspirin ingestion hyperaemia of the mucosa was a constant phenomenon which was confirmed by microscopy of the mucosa. The findings correspond to those obtained by *Muir & Cossar* (24) An increase in the blood flow following damage by aspirin has been demonstrated (1) and might be part of protective, repairing processes. Round cell infiltration in the submucosal layer occurred in all experimental groups, but concomitant submucosal oedema and extension of the infiltration into the mucosa itself occurred only in the aspirin treated animals. The picture was that of a focal acute gastritis. Local oedema and capillary erosion to appear in the vicinity of tablet particles point to a direct damaging effect of aspirin (30 34)

Penetrating lesions imitating chronic gastric ulcers to develop after aspirin have been described only in the paper by *St John et al* (33) Administering the compound in a single dose suspended in methylcellulose, a dose somewhat larger than 160 mg/kg/day was found to be necessary to produce chronic erosions. Tablets swallowed twice a day lead to a more protracted and more direct effect of the compound on the mucous membrane. Simultaneous ingestion of aspirin and acid enhanced the damaging effect. An acid environment determines the degree of damage induced by aspirin (3) This does not necessarily speak in favour of the back diffusion theory (9) An accumulation of salicylate ions inside the cells of the mucous membrane (21) enhanced through a faster absorption at low pH according to pH-partition theory (4) might explain why the damage was more pronounced at low pH. The healing processes observed imitated the usual mucosal epithelium, growing from the circumference and covering the ulcer bed. No glandular proliferation in the submucosa was seen (33) A reduction of mucous substance was demonstrated involving acid as well as neutral mucopolysaccharides. The significance of this finding is hard to evaluate. Mucus acts as a lubricant it protects the mucous membrane against mechanical trauma, but it does not interfere with the diffusion of hydrochloric acid and has no buffering power itself (11) In one *in vitro* study some inhibition in proteolytic activity of pepsin was produced by adding acid mucopolysaccharide (21) In gastric mucus from dogs, *Hakkinen et al.* (15) have found a sulphoglycoprotein that was secreted by the superficial mucous cells and selectively inhibited by aspirin. In mucosal specimens collected from man, none showed any concentration of intracellular sulphated glucoproteins (35) Together with mucous secretion, a secretion of bicarbonate takes place which increases the pH and decreases the activity of pepsin. If bicarbonate secretion is inhibited by aspirin, it might be of greater significance in the pathogenesis than inhibition of mucous secretion itself

Any effect of aspirin on ATP-ase activity was not demonstrated. If aspirin uncouples oxidative phosphorylation in the mucous membrane, as postulated by *Glarborg Jorgensen et al* (14) an increased ATP ase activity was to be expected (17) Any differences in ATP-ase activity were not demonstrated, but this does not exclude a possible increase in activity following aspirin.

NADH reductase and cytochrome oxidase are only 2 groups of oxidative enzymes which transfer hydrogen and electrones in the respiratory chain. The inhibition occurred predominantly in the surface cells. This agrees with the results obtained in the short-term experiment by *Ganter et al.* (1) and with findings in the studies by *Myr n* (25) who

applied the method to mucosal biopsies from patients with gastritis. Of special interest is the finding of decreased reactions in deeper layers of mucous membranes from aspirin treated rats. This finding indicates an impairment of the cellular metabolism, even in the deeper layers of the mucous membrane.

The initial effect of salicylate damage to the stomach mucosa seems to be an impairment of oxidative enzymes followed by a cellular reaction (focal gastritis) reflecting reversibility. Irreversibility of the damage is finally demonstrated through the occurrence of true ulceration, indicating insufficiency of repair processes.

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CENTRAL PONTINE MYELINOLYSIS

A Case Report

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The first case of central pontine myelinolysis in Scandinavia is presented. A premortem diagnosis was established on the basis of the history of chronic alcoholism, the picture of severe electrolyte disturbance and the development of the neurological syndrome. The postmortem findings verified the clinical diagnosis. A possible aetiological mechanism is postulated. The cause is believed to be damage to the energy dependent electrolyte transport across the cell membrane, resulting in cytotoxic oedema and death of the oligodendroglial cells of the brainstem which are particularly vulnerable.

Key words: Pontine myelinolysis, central.

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Central pontine myelinolysis in alcoholics and malnourished patients was first described by Adams and his associates in 1959 This entity has since then been the subject of several case reports from various countries though as far as we know not from Scandinavia. Onset of the disease is acute and it runs a progressive course to end fatally within weeks or months. Pathologically it is characterized by a symmetrical demyelinated lesion evolving from the central pontine base. The aetiology and pathogenesis of central pontine myelinolysis is still unknown. The purpose of this report is to describe the case first encountered in Scandinavia and to relate the clinical signs and symptoms with postmortem findings since the autopsy verified the proposed clinical diagnosis. Common characteristics of the aetiology and possible pathogenetic causal factors of this disease will be discussed.

CASE REPORT

Clinical course A 54-year-old man was admitted to the Sahlgren Hospital, Gothenburg, on May 10 1974 because of general muscle weakness. The patient was a chronic alcoholic with asymptomatic hypertension, known to be manifest for a *few* years, and since then treated with alprenolol and chlortalidon, with the addition of KCl. According to his relatives, the patient had probably daily been drinking large quantities of alcoholic beverages prior to admission and he had not taken the prescribed dose of KCl. Initial laboratory data revealed hypocalaemia 1.6 mEq per litre, which explained the muscle weakness, hyponatraemia, 105 mEq per litre, and a moderate hypochloraemic alkalosis. There was no hypoproteinaemia. After correction of the electrolyte imbalance, the patient was allowed to get up on May 15

Neurological signs of brain stem lesion first appeared on May 17 The patient developed disturbance of gait, swallowing, and linguistic performance. He became incontinent and the body temperature rose to 38 C. During the following days these symptoms progressed. On May 20 the patient was unable to swallow and became drowsy

miosis, especially on the left side, was in evidence, though reaction to light as well as fixation were normal. There was a slight paresis of the tongue, left-sided facial paresis and slight spastic tetraplegia which was most pronounced on the right side. On May 21 these symptoms were more pronounced. Bilateral facial paresis was suspected. Corneal sensibility was normal. The next day he became unconscious, eye fixation was absent and on pain stimulation, bilateral decerebrate posture was noted. The pupils were of identical size but the left corneal reflex was weaker than the right. Cold sweat was noted. On pain stimulation flexion of the upper and lower extremities was noted on May 27. Furthermore, hyperventilation, tremor of the head, and left-sided gaze paralysis were noted. It was not possible to communicate with the patient although he looked up spontaneously. As from June 5 there was steady deterioration. The pupils were fixed. On June 11 the systemic blood pressure dropped and the rectal temperature rose to 40 C. The patient died on June 12 26 days after the first signs of pontine disease had appeared.

Clinical investigations. Echo-encephalography, X-ray pictures of the skull, and vertebral angiography performed on May 20 showed no abnormality. Two days later on May 22 lumbar puncture did not reveal any cells, the protein content was 48 mg per 100 ml, and liquor electrophoresis showed slightly increased alpha 2 fraction. The serum electrophoresis was normal. Lumbar puncture repeated on May 31 showed no cells and revealed a protein content of 34 mg per 100 ml. Liquor electrophoresis exhibited signs of a marked degenerative process with markedly increased alpha 2, tau and sialidase. Electroencephalogram recorded on May 21 showed triphasic potentials which were interpreted as subcortical activity. The trial, repeated on May 27 showed no triphasic activity. Isotopeencephalography was normal on June 4. Folic acid and vitamin B 12 levels in serum were normal. Liver function tests showed nothing remarkable. The erythrocyte sedimentation rate gradually increased.

Treatment. Injections of vitamin C and vitamin B, including thiamine, but excluding B_1 and folic acid were started on May 20 though without effects. Penicillin and corticosteroids were given from May 21 but any signs of clinical improvement did not occur. The electrolyte imbalance in serum was corrected before signs of the pontine disease appeared.

General autopsy findings. The heart, which weighed 400 grams, was dilated but without any signs of hypertrophy. The coronary arteries were moderately atherosclerotic without obliterations. The bronchi showed purulent infection. Both lungs were sites of multiple partly confluent, bronchopneumonic foci. The pleural membranes were covered by a fibrinous exudate. Pancreas and the biliary tracts appeared normal. The liver was swollen and the cut surfaces were of a pale yellowish colour. A moderate congestion in the liver was noticed. Except for the prostate gland which contained some pea-size adenomas, the uro-genital organs appeared normal.

Microscopic examination of the liver revealed fatty changes and heavily lipid-laden cells, predominantly in the centre of the lobules. The portal fibrous tissue was slightly thickened and contained a moderate number of lymphocytes. Slides from the lungs showed large foci of inflammation with exudative consolidation containing some abscess formations.

Neuropathological Findings

Macroscopic appearances. The brain, brainstem and cerebellum weighed altogether 1600 grams. The brain showed pronounced oedema with a tense dura, flattened convolutions and narrowed sulci. Both unci were indented. Despite the oedema, gyral atrophy of the frontal lobe was evident. The pons was heavily swollen, the transverse diameter being 50 mm (normally 27 to 34 mm according to *Stepanenko* (1961)) and of soft consistency. Vermis cerebelli was atrophied. Cerebellum appeared otherwise normal. The leptomeninges were thin and translucent. Moderate atherosclerosis was found in the basilar artery and in the proximal parts of both middle cerebral arteries. In the angle between the anterior communicating artery and the left anterior cerebral artery an unruptured saccular aneurysm of 3 mm was found. The major tributaries of the great vein of Galen were entirely normal.

In frontal sections of the brain, cortex and the white matter appeared normal. The lateral ventricles were slightly widened. Basal ganglia, amygdaloid nuclei, and the hippocampus formations appeared all normal. Both mammillary bodies were found to be of normal size. Corpus callosum showed normal width and no lesions. No haemorrhages were found around the third ventricle. Sections through the pons showed an extensive soft discoloured area in the central pontine base leaving uninvolved only a three to four mm thick ring at the periphery of the pons. The lesion extended from the level of the anterior medullary velum to the level of the lateral recesses of the fourth ventricle leaving mesencephalon and the most caudal part of the pons as well as the cerebellar peduncles of the pons uninvolved. Sagittal sections through the cerebellum showed marked atrophy of vermis, predominantly of the central lobule and culmen. Cerebellum appeared in other respects not remarkable. The spinal cord appeared normal, the spinal roots and ganglia were of ordinary size.

Microscopic appearances. Cerebrum. In various regions of the cerebral cortex there was a moderate

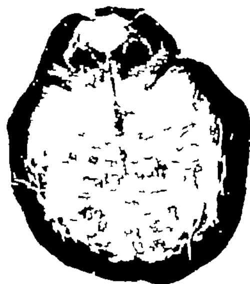


Fig 1 Section of rostral pons, showing a large symmetric area of demyelination. Luxol 2.5 ×

intracerebral oedema and neurones showing changes of the so-called ischaemic type. In the frontal cortex a diffuse loss of neurons was noted. In silver-stained paraffin sections, according to *Palmgren* (1948) and in sections stained for myelin with Luxol, the axons and myelin sheaths appeared unaffected. The hippocampal pyramidal cell layer exhibited a slight loss of neurones in Sommer's sectors and in the end-plate. The thalamus, caudate and putamen showed normal neuronal population and normally stained myelin. No focal lesions, haemorrhages or vascular changes were observed, indicating Wernicke's encephalopathy. The corpus callosum appeared microscopically well preserved without loss of myelin or focal lesions. Both mammillary bodies exhibited an apparently normal histology.

P Numerous sections through the pons revealed a symmetrically extended area completely devoid of myelin, involvement being maximal in the rostral part of the pons (Fig 1). The nerve cells of the lesion were well preserved and without any apparent loss in number (Fig 2). In Palmgren stain, the axis cylinders appeared reduced in number. Along the axons a paucity of intrafascicular oligodendroglia was noticed. In Palmgren stain, the oligodendroglial cells showed pyknotic nuclei and shrunken cell bodies (Fig. 3) as compared with those in unaffected areas of the pons. Reactive astrocytes were almost absent in and around the demyelinated areas. Numerous lipid-laden macrophages were present in the lesion and at its borders. N petechial haemorrhages were present. The intrapontine arteries and veins were unaffected.

Cerebellum. Sections from vermis showed a marked atrophy of the white matter, a pronounced loss of Purkinje cells and a narrowed granular zone. The loss of Purkinje cells was beyond the extent which could be expected to be due to a final ischaemia.

Medulla oblongata and the spinal cord. Sections through the medulla oblongata and the cervical segments of the spinal cord showed ordinary myelinated longitudinal nerve tracts and unaffected neuronal populations of the grey matter.

Clinical-Pathological Correlation

In an attempt to correlate the clinical and pathological findings in the present case, we may assume the following mode of evolution of the pontine lesion.

The initial symptoms indicate an involve

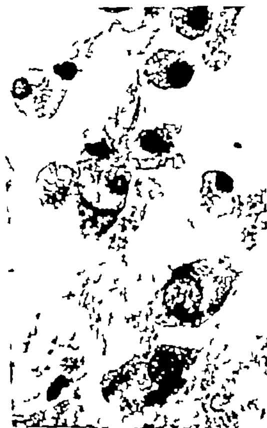


Fig 2 High magnification of the pontine lesion with lipidladen macrophages, intact neurones and demyelination. Luxol 300 ×

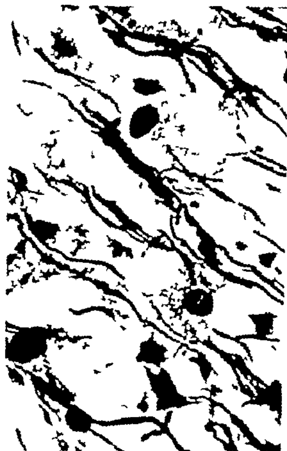


Fig 3 A portion of the pontine lesion with moderate distortion of the axis cylinders. Their integrity is for the most part preserved. Oligodendrocytes show necrosis. Palmgren stain. 300 ×

ment of the medial pyramidal tracts gradually extending laterally The miosis and anisocoria may be explained by a lesion of the sympathetic fibres in the paramedian reticular substance, most pronounced on the left side The pareses of the tongue and palate were probably supranuclear The facial paresis was first noted on the left side. This together with the initial, mainly right-sided spasticity supports the assumption that the extension of the lesion is most pronounced on the left side. The gaze paralysis to the left may be explained by a lesion of the paramedian reticular substance dorsally and to the left In the same manner the hyperventilation may have been caused by a lesion of the paramedian reticular substance dorsally between mesencephalon and the middle third of the pons.

Consequently it may be assumed that the pontine lesion was first restricted to the central-ventral part of the pars basilaris pontis, then gradually involving additional structures in the pons, extending dorsally in the paramedian reticular substance and laterally predominantly to the left.

DISCUSSION

The patient presented in this report was a chronic alcoholic with severe electrolyte imbalance just prior to the onset of pontine symptoms. Vertebral angiography performed during hospitalization showed nothing remarkable and the clinical data revealed normal serum levels of vitamin B 12 and folic acid and a normal liver function. Fatty change of the liver was found at autopsy The basal cerebral arteries exhibited only moderate atherosclerosis and the Galenic venous system appeared normal. Any morphological basis for Wernicke's encephalopathy indicating a deficiency of thiamine was not found.

The pontine lesion involved a symmetric area completely devoid of myelin, necrosis and severe loss of oligodendroglial cells. The neurones and the axis cylinders were well preserved in the demyelinated area. It seems reasonable to assume that the lesion developed as a consequence of damage to the oligodendroglial cells, involving degradation and removal of myelin from the area affected (cf *Paguirigan & Lefken* 1969) The histopathology of the pontine lesion is in accordance with that of central pontine myelinolysis previously described

The atrophy of the grey and white matter in the superior vermis cerebelli also seen in the present case is a common pathological finding in chronic alcoholics (*Victor et al.* 1959) The aetiology of the atrophy of the vermis cerebelli is unknown. Folic acid deficiency has been proposed as a possible aetiological factor and is known to cause a decreased RNA-content of the Purkinje cells in chickens (*Haltia* 1970 In the present case, folic acid deficiency may not have played a primary role as a factor causing atrophy of

the vermis cerebelli since the serum level of folic acid was within the normal range.

The aetiology and pathogenesis of central pontine myelinolysis is unknown. The patients first reported, including those reported by *Adams et al.* (1959) were alcoholics and malnourished patients. Accordingly as further cases were reported, it became evident that the lesion may arise in non-alcoholics and in patients apparently not malnourished.

Several aetiological possibilities of a development of central pontine myelinolysis have been postulated, such as toxic factors and thiamine deficiency related to alcoholic encephalopathy syndromes like Marchiafava Bignami and Wernicke diseases (*Bailey et al.* 1960 *Berry & Olszewski* 1963 *Shernas & Lenty* 1968) vascular mechanisms (*Landers et al.* 1965 *Holdorff* & *Cervos-Navarro* 1971 *Okeda* 1973) and severe disturbances of the electrolyte balance (*Adams* 1962, *Aleu* & *Terry* 1963 *Monteiro* 1971 *Finlayson et al.* 1972) This entity has also been reported to occur in patients with a variety of catabolic states such as infections, renal diseases, and cancer (*Green et al.* 1962 *Shiraki et al.* 1963 *Rosman et al.* 1966, *Finlayson et al.* 1972)

Whenever the possible aetiologic factors involved in central pontine myelinolysis are to be considered it is tempting to assume that at least two factors may be of importance (1) Vitamin deficiency especially thiamine-deficiency since it is frequently present in chronic alcoholics and malnourished patients, and (2) electrolyte disturbances like hyponatraemia, a common laboratory finding in patients reported to have had central pontine myelinolysis.

Further unknown factors of importance may be present, in view of the fact that the deficiencies referred to are rather common clinical findings while central pontine myelinolysis is a rare disease. However as pointed out by *Finlayson et al* (1972) rapid changes of the serum sodium level are more likely to be symptomatic than gradual changes, especially if they occur simultaneously with an adversely affected cellular metabolism.

Thiamine deficiency results in a reduced activity of a number of thiamine-dependent oxidative enzymes. A possible consequence may be a depletion of high-energy phosphate ATP This high-energy compound is required for the active transport of electrolytes across the cell membrane The linked transport of electrolytes, sodium and potassium across the cell membrane however requires an ATP hydrolysing enzyme. This enzyme converts the energy of ATP to electrolyte movements as substrate. Experiments indicate that the enzyme is located to the cell membrane (cf. *Skou* 1965) The activity of the enzyme is dependent on Na and K concentrations in the intra and extracellular phases (*Skou* 1965) In the present case and in reported cases of central pontine myelinolysis, the Na and K concentrations in the serum have been severely reduced. This may have had disadvantageous effects upon the activation of the electrolyte transporting system, especially since Na is the cation with the highest activating potential upon the ATP-hydrolysing enzyme (*Skou* 1965)

A defective transport mechanism of electrolytes across the cell membrane will cause an intracellular accumulation of sodium and fluid i.e. a cytotoxic oedema, in contrast to a vasogenic oedema (*Klatzo* 1967) if more advanced, it may cause cell death.

According to these hypotheses there are two dominant pathogenetic features in central pontine myelinolysis namely thiamine deficiency and electrolyte imbalance, especially hyponatraemia. Separately or together they may result in cell death. However this does not explain the well-defined localization of the pontine lesion, neither does it explain its morphological characteristics including relatively well-preserved neurones and axis cylinders, but necrotic oligodendroglial cells and absence of myelin.

A possible explanation may be derived from the results of some previous experimental investigations. *Bass* (1968) found in the rat that the oligodendroglial cells in the central part of the brainstem are more susceptible to noxious influences than myelin-producing cells elsewhere in the brain. Recent

investigations have demonstrated that experimental thiamine-deficiency in the rat causes lesions strictly localized to the brainstem (*Dreyfus* & *Victor* 1961 *Collins et al.* 1971) Accordingly in the rat at least, the oligodendrocytes of the brainstem are highly vulnerable towards metabolic influences, probably due to their high metabolic rate (*Friede et al.* 1963) which exceeds even that of the neurones (*Hamberger* 1963) If these experimental findings hold true of man also they might underlie the localization and pathological characteristics of central pontine myelinolysis.

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SOME CHARACTERISTICS OF LYMPHOBLASTOID LINES DERIVED FROM HUMAN ASTROCYTOMATA

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Macintyre, E. H., Lindgren, A. & Pontén, J. Some characteristics of lymphoblastoid lines derived from human astrocytomata. Acta path. microbiol. scand. Sect. A, 84 79–84 1976

Two immunoglobulin-producing lines of normal lymphoblastoid cells have been established from cultures of two human astrocytomata. These had the same characteristics as lymphoblastoid lines derived from normal human lymphoid tissue or peripheral blood. It is concluded that the lymphoblastoid lines are derivates of non-neoplastic lymphoid cells (perhaps of the B series) which were present as an infiltrate within the astrocytomata. Furthermore, the slow emergence of the lymphoblastoid cells in culture, their continuing requirement for feeder cells and their production of monoclonal rather that heteroclonal immunoglobulin are attributed to an artefact of growth conditions.

Key words: Astrocytoma, human; lymphoblastoid cell lines

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A lengthy series of cultures in this laboratory from human lymphoid tissue and from peripheral blood was yielded lymphoblastoid cell lines in a high percentage of cases (*Pontén* 1967, *Philipson & Pontén* 1967, *Nilsson* 1971). The characteristics of such lines have recently been defined and a clear distinction drawn between lymphoblastoid lines composed of normal cells and lymphoma lines consisting of tumour cells (*Nilsson & Pontén* 1975).

We report here the culture of lymphoblastoid cell lines from two human brain tumours (astrocytomata). These lines will be shown to be equivalent to cells of lymphoblastoid lines derived from normal lymphoid tissue (*Nilsson* 1971). In this paper will be discussed their source within the astrocytomata and the reasons for the rarity of lymphoblastoid line development in the extensive series of consecutive biopsies of brain tumours cultured in this laboratory.

MATERIALS AND METHODS

Grid and direct cultures were made of 2 human brain tumours (astrocytomata laboratory numbers 119 and 401) following published techniques (*Jensen et al.* 1964, *Pontén & Macintyre* 1968). For tumour 119 samples for grid culture were taken from the centre of the astrocytoma (U 119MO) and from a grossly visible vessel within the tumour (U 119MO). 1 mm cubes of tissue were set up on stainless steel grids covered by gelatin foam (Spongostan) and contained in a petri dish whose

Obtained from A. B. Ferrosan, Malmö.

fluid-gas interface was at grid level (*Jensen et al.* 1964). Grid transfers were made when the area under the grid (2 cm^2) was covered by cells: the grid was moved by forceps to a new petri dish and served as a continuing source of non-trypsinized material for several months. Direct preparations for culture were made without trypsinization by seeding into a 50 mm dish a coarse suspension made from small fragments of tumour by vigorous pipetting. Cells were grown on Falcon plasticware in Eagle's Minimum Essential Medium (MEM) supplemented by 10 per cent foetal calf serum, 100 units of penicillin per ml and 50 µg streptomycin per ml.

The cultures were maintained at 37° C in humidified atmosphere containing 5 per cent CO_2/95 per cent air. Cell transfers were made at confluence using 0.25 per cent trypsin in phosphate buffered saline. F10 medium* was substituted for MEM in the growth medium late in the investigation. Similar procedures were followed for the other astrocytoma, 401, and its cultures designated U-401MGL.

Cells used as feeders included U 119MG astrocytes, U 28 normal human skin fibroblasts (*Pontén & Saksela* 1967) and U 79 CG normal human astrocytes (*Pontén & Macintyre* 1968).

For histology, solid tissue was fixed in 10 per cent formalin overnight; cells in culture were rinsed, then fixed in methanol: acetic acid (3:1) for one hour. The routine stain for solid tissue was haematoxylin-eosin-van Gieson and for cells in culture May-Grünwald-Giemsa. Contamination by PPLO was looked for by exposure of cultures in 60 mm Falcon petri dishes to 5 µc ³H-thymidine for 24 hours and subsequent autoradiography (*Nardone et al.* 1965). Immunoglobulin synthesis was tested using double gel diffusion of concentrated culture supernatants: medium was harvested after three days incubation from cultures containing a total of 5×10^6 cells; cell-free supernatant was concentrated twenty fold and stored at −20 C. (*Phillipson & Pontén* 1967).

The preparation of cells for electron microscopy has been described in considerable detail (*Macintyre et al.* 1972). Briefly, the cells were fixed for 30 minutes in a 1:2 (v/v) mixture of 2.5 per cent glutaraldehyde and of 1 per cent osmium tetroxide in 0.1 M cacodylate buffer at pH 7.4 on ice (*Hirsch & Fedorko* 1968). The initial fixation was followed by several rinses with 0.1 M cacodylate buffer at 0° C, then two rinses with cold 0.1 M acetate buffer pH 6.3 containing 0.25 per cent uranyl acetate (*Hirsch & Fedorko* 1968). The sample was left in contact with the final uranyl acetate solution for 20 minutes at 0 C, then washed with several changes of cacodylate buffer dehydrated through 70, 80 and 97 per cent hydroxypropylmethacrylate in water (allowing 5 minutes per concentration) and finally infiltrated with a series of hydroxypropylmethacrylate and Epon mixtures and embedded in Epon. Sections of various preparations were stained with lead acetate (*Reynolds* 1963) to improve contrast.

RESULTS

1. *Histology*

Sections of tumour 119 (Fig. 1) showed a high grade astrocytoma; this had an unusually heavy infiltrate of round, mononuclear cells, which were concentrated in perivascular regions, had a single eccentric nucleus and moderately rich, deeply basophilic cytoplasm. Tumour 401 was an astrocytoma of lesser grade of malignancy and had only a scattering of round, mononuclear cells. At autopsy the lymphoreticular systems of the 2 patients (119 and 401) showed no abnormal proliferation.

2. *Cell Cultures*

Grid cultures of U 119MG and U-119MGL grew slowly: it took one month for seeded cells to cover the space under the grids. The initial outgrowth was of large, uninucleate cells: these had abundant, palely staining cytoplasm which contained numerous neuroglial fibrils. They were classed as tumour-derived astrocytes (*Shein* 1965). Three and a half months after primary explantation, groups of small, round cells appeared in cultures derived from U 119MGL: these new cells grew initially in broad heaps of 5–50 cells attached to the astrocytes (Fig. 2). Mitoses were frequent within the heaps, and their attachment to the astrocytes was easily broken by vigorous pipetting. As the size of the heaps increased, round viable cells detached into the medium and reached appreciable dimensions in older cultures (1.2 × 10^6/ml). These detached cells could attach to feeder cell layers, but not to free surfaces, and did not survive without feeder cells. Cultures of the other grid, U 119MG, and direct cultures of tumour 119 gave only tumour-derived astrocytes.

* Obtained from Grand Island Biological Company, Grand Rapids, Michigan.

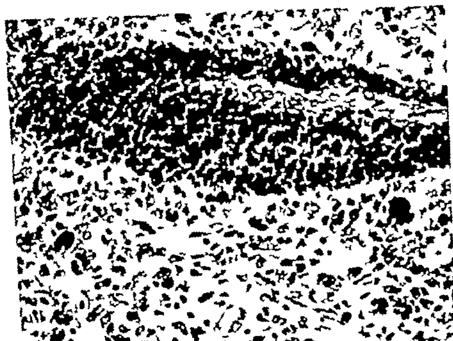


Fig. 1. This section from the original biopsy of tumour shows a vessel, surrounded by many compact round cells and lying within the pleomorphic astrocytoma. The endothelial nuclei (arrow) are swollen and hyperplastic. (Van Gieson × 400.)

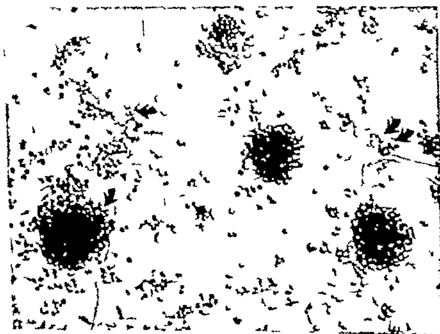


Fig. 2. Cluster of round refractile cells (arrow) are associated with much larger but poorly-defined tumour astrocytes (double arrow). Lh. tissue culture oblique lighting (magn. × 40.)



Fig 3 The plasmalemmal surface of the tumour astrocytes from both U 119MG and U 119MGL cultures carries many long, slender cytoplasmic projections. The granular endoplasmic reticular system (arrow) is well developed, but shows no alignment with relation to the cell surface. (magn. × 10,000.)

Fig 4 The plasmalemmal surface of the lymphoblastoid cells is almost devoid of any protrusions. The granular apoplasmic reticulum is arranged in dilated cysternal arrays (arrow) which lie parallel to the surface of the plasmalemma (magn. × 34 000)

Cultures of tumour 401 grew even more slowly than those from tumour 119 their development was identical with that of U 119MGL otherwise. The cultures were designated U-401MGL.

The continued growth of round cells in U 119MGL cultures was associated with a progressive diminution in the numbers of astrocytes within the same culture, and approximately 8 weeks from the initial appearance of round cells, the astrocytes had disappeared from the U 119MGL cultures. The round cells could not survive without access to feeder cells, and were saved by substituting as feeders astrocytes from cultures derived from tumour 119 which had not yielded round cells (U 119MG grid and U 119MG direct cultures) Other suitable feeder cells were U 2S normal skin fibroblasts (*Pontén & Saksela* 1967) and normal human astrocytes.

In all cases after mixture, the feeder cell population numbers dropped while the round cell population continued to grow With time, fewer and fewer cells were needed to maintain the attachment of the round cells to the growth surface, as many as 200 round cells being attached to one feeder cell. PPLO was absent from the astrocyte/round cell cultures, as adjudged by the absence of grains over the cytoplasm or external to the cells and presence of grains over nuclei after exposure of the cultures to H^3 TdR and subsequent autoradiography (*Nardone et al.* 1965)

Late in the investigation U 119MGL and U-401MGL cultures were placed in a richer growth medium, F10 The round cells then grew freely without feeder layers.

3 *Sections of Grid Primary Explants U 119MG and U 119MGL*

Seven months after primary explant *in vitro* sections were made of grids U-119MG and U 119MGL. Both showed pleomorphic astrocyte tumour tissue Grid U-119MGL alone had groups of large round cells.

4 *Immunoglobulin Production*

Immunoglobulin production was tested from feeder cell layers U 119MG U2S and U 79CC as well as from the round cell cultures U 119MGL and U-401MGL (*Philipson*

& *Pontén* 1967) The feeder cultures synthesized no immunoglobulins lines U 119MGL and U-401MGL produced a monoclonal immunoglobulin-IgG Round cells in cultures U 119MGL and U-401MGL will henceforth be designated lymphoblastoid cells (*Philipson* & *Pontén* 1967 *Nilsson* & *Pontén* 1975)

5 *Chromosomes*

No abnormality of morphology was seen in chromosome spreads from U 119MGL cultures. The cells were diploid. The tumour derived astrocytes from U 119MG cultures were heteroploid.

6. *Ultrastructure of U 119MG and U 119MGL Cultures*

The tumour astrocytes in both U 119MG and U 119MGL cultures were large cells (cf Fig 2) They had a very irregular cytoplasmic border from which numerous long slender projections protruded (Fig. 3) There was no special alignment of the granular endoplasmic reticulum (Fig 3, arrow) to the plasmalemmal surface. The lymphoblastoid cells (Fig 4) had an essentially smooth plasmalemma. Linear arrays of dilated cysternae lay parallel to the surface of the plasmalemma (Fig. 4 arrow) as in immunoglobulin-producing lymphoblastoid cells (*Hummeler et al* 1966)

DISCUSSION

U 119MGL and U-401MGL lines have two features which do not accord with the definition (*Nilsson* & *Pontén* 1975) of lymphoblastoid lines derived from normal lymphoid tissue These are a requirement for feeder cells and the production of monoclonal rather that polyclonal immunoglobulin. However the feeder requirement is not absolute and is overcome by substituting a richer growth medium [Conversely if normal lymphoblastoid cell lines grown in the usual conditions in a rich medium are transferred to MEM they also require feeder cells, which they do not in the richer media, i.e. they have the need for feeder cells imposed upon them by the conditions of culture (*Nilsson* 1971) Also monoclonal and not polyclonal immunoglobulin production is the usual finding where lymphoblastoid lines from normal tissue are grown in MEM medium instead of F10 or other rich media (*Nilsson* 1971)] It is concluded that the anomalies of feeder cell requirement and early monoclonal synthesis shown by the U 119MGL and U 401MGL lines are attributable entirely to the use of a medium (MEM) which is not optimal for normal lymphoblastoid cells, and these anomalies result from an artefact of growth conditions.

Lymphoid tissue is routinely cultured here in a richer medium than MEM (*Nilsson* 1971 *Nilsson* & *Pontén* 1975) often F10 Astrocytes grow poorly in F10 and well in MEM (*Macintyre Nilsson* & *Pontén* unpublished observation) MEM is therefore used routinely in cultures of astrocytes. This probably accounts for the rarity of isolation of lymphoblastoid lines from the lengthy series of astrocytoma cultures in this laboratory (*Pontén* & *Macintyre* 1968)

We consider that U 119MGL and U 401MGL lymphoblastoid lines were derived from the round cells which infiltrated the tumour and grew therefore from non-neoplastic cells present within the primary tumour explant. Their precursor cell is probably of B cell type, at a stage between the small B lymphocyte and the plasma cell but this requires further study (*Nilsson* 1971 *Nilsson* & *Pontén* 1975) The round cells infiltrating astrocytomata are therefore lymphoid and can form permanent lines of immunoglobulin-synthesizing normal lymphoblastoid cells in vitro

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HEALING OF A CRUSH INJURY IN RAT STRIATED MUSCLE

3. A Micro-angiographical Study of the Effect of Early Mobilization and Immobilization on Capillary Ingrowth

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Järvinen, M. Healing of a crush injury in rat striated muscle. 3. A micro-angiographical study of the effect of early mobilization and immobilization on capillary ingrowth. Acta path. microbiol. scand. Sect. A, 84: 85–94, 1976.

Vascular ingrowth into the injured area of a partially crushed gastrocnemius muscle was studied by micro-angiography 2 to 14 days after the trauma in 40 rats treated by mobilization or immobilization. In mobilized muscles, the sprouting of new capillaries occurred more rapidly and intensively than in injured muscles treated by immobilization. The sequence of events was found to be slightly delayed if mobilization was started after immobilization for two days. The speed and intensity of tissue repair, as studied histologically, was directly correlated with vascular ingrowth, especially during the first week.

Key words: Striated muscle; crush injury; healing; micro-angiography; treatment; mobilization; immobilization.

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The repair of injured muscle occurs by simultaneous formation of connective tissue scar and regeneration of muscle tissue (c.f. *Betz et al.* 1966). The speed and intensity of the reparative processes are strongly dependent on the physiological state of the injured muscle during healing. Early activity after a trauma leads to a more rapid disappearance of degenerative changes, followed by more rapid and intensive scar formation and muscle regeneration, as compared with immobilized injuries (*Kvist et al.* 1974, *Järvinen* 1975). The sequence of events during regeneration are found to depend especially upon the vascularity in the injured area as the formation of myotubes occurs only in close connection with capillaries (*Carlson* 1970). In the study of ingrowth of vascularity into muscle wounds, micro-angiography has been used by *Schoefl* (1963) who noticed that new capillaries sprouted from the surviving trunks of blood vessels towards the centre of the injury. As regards the rates of morphological features of repair and growth of capillaries in injured muscles, any comparative studies have not yet been published.

In order to study the background of morphological differences to be demonstrated in repaired muscle after mobilization or immobilization treatment (*Kvist et al.* 1974, *Järvinen* 1975), the ingrowth of new capillaries into healing tissue is studied and compared

during treatment according to these two principles.

MATERIALS AND METHODS

Experimental Animals

Forty male rats of the Wistar strain were used in this study. At the time of traumatization their average age was 22 weeks (range 20–34 weeks) and their average body weight 297 g (range 240 to 350 g). The animals were housed in wire (mesh) cages and received laboratory chow (Hankkija, Finland) and water *ad libitum*.

Traumatization of the Animals

The constant contusion injury using a blunt spring-loaded hammer was induced to the left calf of each animal under light ether anaesthesia. The traumatization procedure has been described in detail earlier (*Järvinen & Sorvari* 1975). The time of traumatization is designated day 0 and the consecutive days are called 1, 2 etc.

Post Traumatization Treatment of the Animals

Before traumatization all the animals were taught to run on the treadmill and thereafter divided into three groups.

GROUP MO (16 rats): the animals were exercised each day beginning on day 1 in a motor driven treadmill with an inclination of 15 at a speed of 40 cm/sec. The training time on the first day consisted of two 20-minute-periods, on day 2 of two periods of 30 minutes, thereafter they were trained for 60 minutes once daily. The same mobilization programme was also used in our earlier studies (Kvist *et al.* 1974, *Järvinen* 1975). During the experimental period the animals were weighed daily.

GROUP IM2 + MO (8 rats): the animals were treated with a softened plaster cast around the contused leg for two days after traumatization and mobilized thereafter as in GROUP MO.

GROUP IM (16 rats): the animals were treated by immobilization. Immediately after the trauma, the contused leg was immobilized by a softened plaster cast. This procedure in the care of immobilized animals has been described earlier in detail



Fig. 1. Micro-angiogram 2 days after the injury. GROUP MO. The injured area is totally non-vascularized. Little evidence of capillary sprouting is seen at the proximal border of injury (to the right in the figure). 60×



Fig. 2 Micro-angiogram 2 days after the injury GROUP IM. The non-vascularized injured area is similar to that in Fig. 1. No evidence of capillary sprouting from the survived stumps of vessels is seen at the borders of injury. 60 ×

(*Järvinen* 1975). In GROUPS MO and IM the animals, 4 on each occasion and in each group, were studied on days 2, 5, 7 and 14, those in GROUP IM2 + MO on days 7 and 14.

Micro-angiographic Technique

On days 2, 5, 7 and 14 the vascular anatomy of injured muscle was outlined by a modification of the technique used by *Wendelin* and *Lindgren* (1970) and *Chaplin* (1974). The animals were anaesthetised with intraperitoneal pentobarbitone sodium (Nembutal® Abbot, England), the thorax was opened and 5000 units of heparin (Heparin® Medica, Finland) was injected into the left ventricle of heart. After a short interval, a polyetene catheter with an internal diameter of 0.85 mm was tied into the left ventricle and physiological saline solution, temperature 32–37° C, was perfused at a constant pressure of about 100 mm Hg (the container was positioned about 1.3 m above the rat). After 4–5 min, the perfusion was continued using a warm, 10 per cent radiopaque yellow suspension (Cromopaque® Damancy & Co Ltd, England) for about 20–30 minutes, since the flow became slower. Thereafter the perfusion mixture was changed to 10 per cent Cromopaque diluted in 10 per cent neutral buffered formalin and the rat was totally immersed in a 10 per cent formalin solution.

The infusion was continued until it ceased, practically overnight. Thereafter the left leg was exarticulated at the hip and kept for about 7 days in a formalin solution. The triceps surae muscle was removed from the bone and fixed for an additional 2 days in formalin.

The tissue samples were dehydrated in graded alcohols, cleared in xylene, and embedded in a medium containing paraffin and plastic polymers (Fibrowax, supplied by Bethlehem Trading, Göteborg, Sweden). Serial 500 μm sections were then cut sagittally from muscle blocks. The sections were placed in contact with the photographic emulsion (Kodak High Resolution Plate, USA). A Machlett AEG-50 roentgen tube with a 1.5 mm focal spot and equipped with a 1.5 mm beryllium window was employed; this was run at 30 kV. The film-focus distance was 0.5 meter.

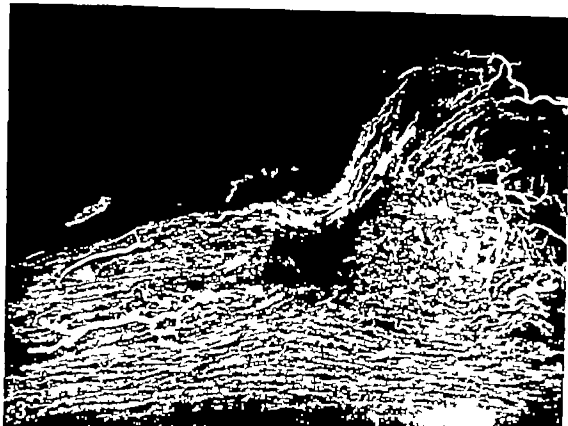


Fig 3 Micro-angiogram 5 days post-traumatization, GROUP MO Intensive ingrowth of new capillaries from all borders, most extensively from the proximal border of injury and towards the non-vascularized centre of the injury is found, 60 ×

Sections measuring 6 μm and obtained from the sagittal midline of the injured area were prepared and stained according to the van Gieson-haematoxylin method to be used for microscopical examination.

The size of the injured area and the location of the capillary pattern were identified in the micro-angiograms and also verified microscopically on the basis of the corresponding histological section. Each observation was made twice without knowledge of the group to which the specimen belonged. The width of the non-vascularized area was estimated with respect to the high power (40 ×) field of the microscope (about 3.5 mm in diameter) and the relative amount of capillaries was estimated semiquantitatively by scoring, using numbers from 3 (abundant) to 0 (none) These measurements were made on one micro-angiogram from each animal.

The amount of myoblasts, myotubes and capillaries in the middle and in the transitional zone of the injury were also estimated semiquantitatively on the basis of histological sections taken from the sagittal midline of each injured muscle by scoring from 0 (none) to 3 (abundant)

The semiquantitative scoring system according to estimate used in this study was based mainly on that used in an earlier study of a larger material (*Järvinen* 1975)

RESULTS

Day 2. The area without any filled vessels and identified as the injury was always quite constant (about 5 mm in height) (Figs. *1* and 2) The vessels close to the injury were better filled with contrast medium than those far from it. Beyond the ends of the crushed vessels, no leakage occurred. Some sprouting of new capillaries at the sites of surviving vessels was observed in GROUP MO

Histological differences between GROUPS MO and IM were similar to those described



Fig 4 Micro-angiogram 5 days post traumatization, GROUP IM. The capillary ingrowth is negligible. At the distal border of the injury (to the left in the figure) a large leakage of contrast medium is seen, 60 ×

earlier in detail (*Aro et al.* 1974 *Järvinen* 1975) the filled capillaries at the borders of an injured area being more numerous in GROUP MO than in group IM (mean score 1.5 and 1.0 respectively) In the centre of the injury filled vessels were totally lacking.

Day 5 The capillary proliferation at the borders of the injury was by far more prominent in GROUP MO than in GROUP IM (Figs. 3 and 4) The new capillaries penetrated the granulation tissue beginning at all the borders of the injury and directed towards its centre in GROUP MO some had already reached the middle of the crushed area. In one case in both groups, a large leakage of contrast medium (c.f. Fig. 4) was observed at this stage.

Histological study showed distinctly the close connection between myotubes and filled capillaries. Abundant capillaries (mean score 3.0) and myotubes close to each other were observed at the borders of the injured area in GROUP MO (Fig 5) On the other hand, in GROUP IM only a few myotubes were found and filled capillaries were less numerous (mean score 1.5) (Fig 6)

Day 7 In general, the middle of an injury without vascularity would appear only as a narrow line surrounded by radially growing capillaries. In addition, the filling of the new capillaries in the granulation tissue was better than that in muscular tissue far from the injury The amount of new capillaries was abundant in GROUP IM2 + MO in GROUP MO their number seemed to be reduced as from day 5 and the intergroup differences were as a rule diminished from that time. The visualized vessels in muscle

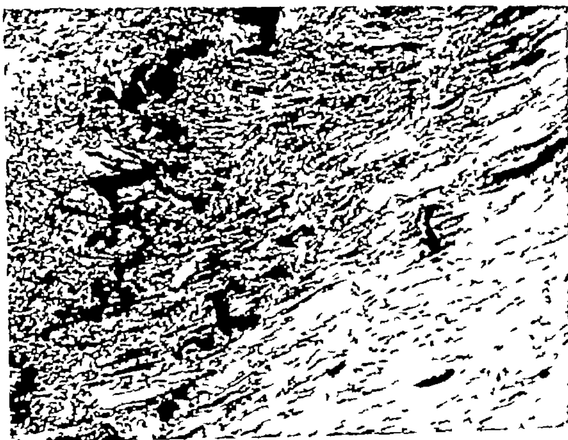


Fig. 5. Histological picture from proximal transitional zone of injured area, 5 days after trauma, GROUP MO. Numerous myotubes in close connection with filled capillaries (black) among granulation tissue are seen. Van-Gieson haematoxylin, 110 ×

tissue had generally increased in amount, especially in the mobilized muscles, after day 5 more prominently in plantaris and soleus muscles.

Day 14. The area without vascularity in the middle of the injury had totally disappeared and the density of the vascular bed was reduced, being similar to that elsewhere in the uninjured part of the muscle. The radial orientation of vessels connected with the injury was typical due to the contraction of the scar and to the maturation of repaired tissue. The vascularity in the repaired area of the mobilized muscles was found to be more abundant than that in immobilized muscles (Figs. 7, 8 and 9).

DISCUSSION

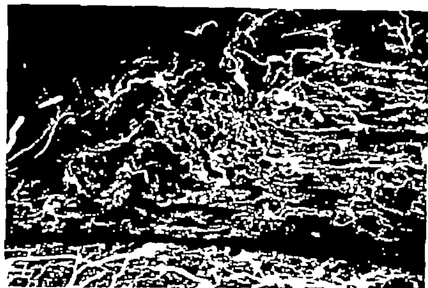
Ingrowth of new capillaries is one of the main features of the proliferation phase in wound healing (*Ross* 1968). In striated muscle the new capillaries are produced by sprouting from the stumps of crushed vessels at the borders of the injury and the migration of endothelial cells precedes the growth of fibroblasts (*Schoefl* 1963). Blood pressure, low oxygen tension, changes in ground substance, and metabolic products are assumed to initiate capillary growth (*Schilling* 1968). *Remensnyder & Majno* (1968) demonstrated clearly that oxygen tension in the central avascularized area of the muscle injury was strikingly low and that the hypoxic gradient in wounds disappeared when the new blood vessels had grown completely across the

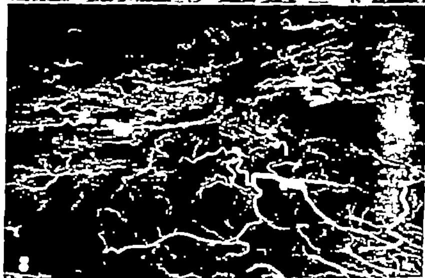


Fig. 6. Histological picture from proximal transitional zone of injured area, 5 days after trauma, GROUP IM. Some small myotubes and only a few filled capillaries are found among granulation tissue. Van-Gieson-haematoxylin, 110×

wound. The fragility of new capillaries in the injured area demonstrated by *Schoefl* (1963) is assumed to be connected with the low oxygen tension in tissue (*Remensnyder* & *Majno* 1968). Therefore the method used in investigations of the vascular bed of injured muscle should be as physiological as possible to avoid the artifactual leakage during micro-angiographic procedures, although leading to the sufficient filling of open vessels (*Schoefl* 1963). *Hendelin* & *Lindgren* (1970) concluded that the best method by which vessels may be filled with a view to demonstrating capillaries is to maintain the temperature of the tissue to be studied and the contrast medium within physiologic limits. This is possible when the animal is anaesthetized and dies during the infusion. If the degree of filling of capillaries was evaluated by the alkaline phosphatase method used earlier (*Kormano* & *Reijonen* 1973) only about 20–30 per cent of all capillaries in the gastrocnemius muscle would be properly filled. However as many capillaries in the gastrocnemius muscle generally are closed (*Hudlická* 1973) and the vascularity near the injury in every case was well demonstrated, the method used can be regarded suitable in studies of the capillary ingrowth into an injured area.

Combined micro-angiography and histology afford additional information about the relationship between the microscopic structure and appearance of the capillary bed in the micro-angiograms (*Kormano* 1970). Besides, the contrast medium Cromopaque is




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found to be distinctly demonstrable in histological sections stained by van-Gieson method (*Hendelin & Lindgren* 1970)

The results of this study confirm earlier observations (*Carlson* 1970) namely that there is a close geographic relationship between the ingrowth of vascularity and the initiation of the regenerative process, especially the formation of myotubes (Figs. 5 and 6)

More rapid and intensive capillary growth in mobilized muscles during the proliferation phase of healing seems to be associated with a more rapid and intensive regeneration of muscle and formation of connective tissue as demonstrated earlier (*Kvist et al.* 1974 and *Järvinen* 1975)

The fact that the muscular blood flow is increased both during mobilization and also after immobilization for more than one week (*Hudlická* 1973) serves to explain why a greater number of capillaries are filled, especially in the deeper part of calf muscles, after treatment for one week. The better filling of vessels near the injury has been assumed to indicate some vasodilatation caused by the injury (*Karppinen & Myllärniemi* 1967). The functional term, vasodilatation is not suitable when micro-angiographical observations are to be demonstrated as the procedure itself exerts an effect on the actual vascular diameter (*Lundskog et al.* 1968) and, if formalin is used as fixative, it may also lead to artifactual changes in vascular diameter (*Suoranta & Kormano* 1974). However the observations by *Remensnyder & Majno* (1968) namely that oxygen tension immediately surrounding the injury is high, thus suggesting a persistent functional hyperaemia and arterialization of the neighbouring vasculature is in support of the postulation that the better filling of vessels near the injury is due to functional vasodilation. As striated muscle is known to be a tissue where blood flow can be enormously changed under different physiological circumstances, the healing of striated muscle injury serves as a suitable model in studies of the effects of blood flow on the repair process during wound healing.

Fig. 7. Micro-angiogram 14 days after the injury GROUP MO. Completely vascularised injury with radial growth vessels is seen in the middle of figure. Abundant filled vessels in the more deeply located soleus (under part of figure) are well demonstrated, 60×

Fig. 8. Micro-angiogram 14 days after the injury GROUP IM. In the middle of the injured area is still non-vascularised area. The vascular density in deeper muscles is less than that in GROUPS MO (Fig. 7) and IM2 + MO (Fig. 9). 60×

Fig. 9. Micro-angiogram 14 days after the injury GROUP IM2 + MO. Completely vascularised injury with numerous capillaries is seen in the middle of the figure. Also in the more deeply located soleus there is pronounced filling of the vasculature. 60

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GLYCOSAMINOGLYCANS AND PROTEOGLYCANS OF HUMAN BONE TISSUE AT DIFFERENT STAGES OF MINERALIZATION

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Engfeldt, B. & Hjerpe, A. Glycosaminoglycans and proteoglycans of human bone tissue at different stages of mineralization. Acta path. microbiol. scand. Sect. A, 84 95–106 1976.

Using ultracentrifugation, compact bone from a growing 15 years old man was separated into three fractions with different degrees of mineralization. The finely powdered material was fractionated in a density gradient of acetone and bromoform in varying proportions. The glycosaminoglycans were isolated after papain digestion of the tissue fractions. The glycosaminoglycan elution profiles from CPC-cellulose microcolumns indicated the presence of chondroitin-4-sulphate and possibly minor traces of hyaluronic acid. The degree of sulphatation was the same in all three fractions, while the total amount and molecular size of the glycosaminoglycans decreased somewhat with increasing degree of mineralization. The glycosaminoglycans of the tissue fractions were also extracted with 4 M guanidinium chloride and then purified chromatographically on DEAE. Gel chromatography of the isolated material showed three different peaks, two probably representing low molecular weight proteoglycans. The proportion of intermediate molecular size material increased with increasing degree of mineralization. The results are discussed briefly in relation to the mineralization process.

Key word: Bone mineralization; glycosaminoglycans; proteoglycans.

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Since the 19th century it has been known that bone contains small amounts of sulphate (*Morner* 1897) and in 1900 Gies isolated a substance with the same qualities as chondroitin sulphate (CS). Studies by *Rogers* (1949) and *Meyer et al.* (1956) confirmed that chondroitin-4-sulphate (CS-4) was the major glycosaminoglycan (GAG) in cortical bone. *Herbertsson* & *Lefas* (1966) also showed the presence of hyaluronic acid amounting to about 5 per cent of the total GAG.

In cartilage the GAGs are proven to be covalently bound to protein forming proteoglycans (PGs) (*Muir* 1958, *Anderson et al.* 1965). Similar results have been obtained with bone tissue (*Herring* 1968).

The GAGs and PGs are important to the qualities of the ground substance since they exert both steric exclusion and molecular sieving effects (*Laurent* 1968). Furthermore the strongly polyanionic character of the GAGs also makes it conceivable that they take part in the mineralization process

(*Sobel Burger & Nobel* 1960 *Cuervo et al.* 1973)

In compact bone tissue, the newly formed organic matrix of the osteones mineralize rapidly to a level just short of 50 per cent of the most mineralized bone matrix (*Pugliarello et al.* 1970) This formation of the young osteones, comprising 60–70 per cent of the interstitial bone, is followed by a slower process when more calcium salt is deposited This process may proceed until the bone matrix is fully mineralized, i e. 65 per cent of the dry weight is mineral. Thus the growing compact bone consists of osteones at different stages of mineralization, the density of these osteones ranging from 1.85–2.15 g/cm³ depending upon the degree of mineralization (*Engfeldt & Hjerpe* 1974)

Pugliarello et al. (1970) demonstrated a lower hexosamine content in the mineralized part of the osteones than in the nonmineralized osteoid tissue. When further mineralized, the osteones show only a minor decrease in hexosamine contents. A similar difference in GAG contents between nonmineralized predentine and mineralized dentine, has been reported by *Engfeldt & Hjerpe* (1972)

The aim of the present investigation is to characterize and compare the GAGs and PGs of mineralized compact bone tissue at different stages of mineralization. To simplify later studies of mineralizing tissues under pathological conditions, human bone tissue (autopsy material) was chosen.

MATERIAL AND METHODS

Femur was obtained from an autopsy case a 15 year old boy who had died suddenly from an accident. The body had been transferred to the morgue (+4 C) within two hours and the autopsy took place within two days.

Segments of the femur (approximately 0.5 g each) were carefully cleaned on the periosteal and endosteal surfaces. The bone material was ground in liquid N_2, using a *Spex Industries model 6700* Freezer/Mill, and subsequently sieved through 400 mesh sieve (ASTM) The bone tissue was repeatedly milled until no material could be recovered from the upper surface of the sieve. The recovery after grinding and sieving was 90 per cent by weight.

Analytical Methods

To determine the degree of mineralization of the tissue fractions, approximately 1 mg of each fraction was hydrolysed, using 50 µl 6 M HCl at 105 C in sealed pyrex glass tubes for 18 hours. After hydrolysis 2.5 ml of quartz distilled H_2O was added, and aliquots were taken for calcium, phosphate and amino nitrogen analysis. Calcium was determined by chelatometric titration with EGTA (Ethyleneglycolbis/2aminoethylether/NNN' N' tetra acetic acid) using a Marius calcium titrator. Phosphate was assayed according to *Youngburg & Youngburg* (1930) Amino nitrogen was determined using the ninhydrine method of *Moore & Stein* (1948) as described by *Strandh* (1960)

In order to save material for subsequent amino acid analysis, the hexosamine contents of gel chromatography effluents were determined according to the micromethod of *Gardell & Jhonson* (1972) Prior to this determination the materials were hydrolysed for 3 hours in 8 M HCl at 90° C (*Lohmander* personal communication) All other determinations of the hexosamines were performed after 8 hours' hydrolysis with 6 M HCl in a boiling water bath, using the colorimetric method of *Elson & Morgan* (1933) as described by *Antonopoulos et al.* (1964)

Sialic acids from DEAE effluents were determined according to *Svennerholm* (1957)

The sulphate contents were determined after 24 hours hydrolysis of the GAG preparations with 25 per cent v/v formic acid in sealed pyrex glass tubes at 100 C. The analyses were performed using the benzidine method described by *Antonopoulos* (1962)

The protein contents of the Sepharose 6 B effluents from fraction III were determined with the method of *Lowry et al.* (1951) using crystallized bovine albumine (Sigma) as standard.

Tissue Fractionation

To fractionate the tissue powder according to density the density gradient system described by *Engfeldt & Hjerpe* (1974) was used To minimize convection a discontinuous gradient was used, as described below

Bone powder in portions of 0.3 or 0.5 g depending upon the size of the centrifuge tube used, was suspended in an acetone-bromoform mixture with a specific density of 2.02 g cm Over this suspension two acetone-bromoform mixtures with specific densities of 1.96 and 1.75 g cm respectively were layered. In the small tubes (used in a Spinco SW 25 1 rotor) the mixture volumes were 10 15 and 6 ml respectively counting from the bottom of the tube while in the large tubes (used

The authors are indebted to Dr C Antonopoulos for this analysis.

in a Spinco SW 25.2 rotor) the corresponding volumes were 20 .5 and 12 ml. The gradients were centrifuged at 35.000 × g and at +6 C in a Beckman L-65B ultracentrifuge.

The tubes were emptied with the aid of an acetone-bromoform mixture with a specific density of 2.02 g/cm which was infused (1.4 ml/min) through the wall of the centrifuge tube, just above the pellet, which was formed of the heaviest bone particles. Fractions were changed after 1.5 10 and 20 min for the small tubes, while for the large tubes the corresponding time intervals were 3.75 19.25 and 37 min, respectively. Between each fraction a few drops were taken for simultaneous refractometry to check the density limits of the fractions. In order to check the discontinuity one gradient was emptied, determining the densities at one minute intervals. The corresponding fractions from the different centrifuge tubes were pooled, giving three fractions, designed fraction I–III, and the pooled pellets gave fraction IV. The overall recovery was 70 per cent. The first fraction, amounting to only 74 mg (cf. Table 1) was discharged, while the others were saved for further analyses.

Isolation of GAG and PG

Samples (1.1 1.5 and 1.1 g) from tissue fractions II III and IV respectively were digested with papain as described by *Hjertquist* (1964). The digests were dialysed for 24 hours against a 0.05 M phosphate buffer at pH 7.0 and the GAGs were precipitated when cetylpyridinium chloride (CPC) was added. The precipitates were dissolved in 1 ml n-propanol (60 per cent / in water and containing 0.5 per cent w/v CPC) and the GAGs were precipitated in the form of sodium salts when a 9:1 mixture of ethanol and 25 per cent w/ sodium acetate in water was added. The precipitates were washed twice in ethanol nd once in diethylether and finally dried.

The remaining tissue powders were used for extraction of PGs in 4 M guanidinium hloride, pH 5.8, according to *Sajder & Hascall* (1969). In addition, the solution contained 0.2 M EDTA. 2 30 ml of this solution was used per gram dry tissue. The extracts were dialysed gainst 7 M urea and chromatographed on a DEAE-cellulose column according to *Antonopoulos et al.* (1974). The PG fractions, luted with 2 M NaCl in 7 M urea containing 0.05 M tris buffer at pH 6.5 were dialyzed against destilled water and lyophilized.

T determine the degree of extraction, the residues obtained after the extraction of the tissues with guanidinium chloride were washed with water and the remaining GAGs er isolated after papain digestion and precipitation of GAGs as described abov. The total GAG-bound hexosamine contents of these fractions were determined with the Elson Morgan reaction.

Chromatographical Procedures

Isolated GAGs were fractionated on CPC-cellulose microcolumns in order to study the charge density i.e. to estimate the molecular size and/or the degree of sulphatation, columns were eluted with $MgCl_2$ solutions of increasing ionic strength according to *Antonopoulos et al.* (1964). The lowest $MgCl_2$ concentration used was, however 0.4 M by so doing the sensitivity in this region of the chromatogram was increased. To separate different GAGs, if present, CPC columns were also eluted according to the n-propanol/methanol procedure described by *Antonopoulos & Gardell* (1963). To establish the hexosamine identity one column was eluted with 1 per cent w/v CPC, 0.5 M NaCl containing 0.05 per cent w/v CPC and 6 M HCl, taking the final fraction for hydrolysis and subsequent ion exchange chromatography according to *Lohmander* (1972).

In order to separate glycoprotein bound and GAG bound hexosamines, ion exchange chromatography of papain digested material was performed on Ecteola-cellulose (*Axelsson et al.* 1970) or DEAE-cellulose microcolumns measuring 4 × 70 mm. Similar results were obtained with the two resins when eluted with 0.06 M HCl, 0.5 M NaCl, and finally 6 M HCl, taking 2 ml of each solution (*Antonopoulos* personal communication).

To detect keratan sulphate if present in the supernatants after CPC precipitation of GAGs, isopentyl alcohol was used to extract CPC from aliquots of the supernatants (*Axelsson et al.* 1970). The residues were added to DEAE-cellulose microcolumns, and eluted as described above.

The isolated PGs were dissolved in 0.5 M sodium acetate at pH 7.0 and a volume of 0.5 ml (0.5–2.5 mg) was chromatographed on a Sepharose 6 B column, 1450 × 9 mm, and eluted with 0.5 M sodium acetate at pH 7.0 using fractions of 2 ml. For aminoacid analyses B the Sepharose 6 B fractions were pooled dialyzed against water lyophilized nd hydrolyzed in 6 M HCl (Aristar HCl BDH Ltd, England) for 24 hours at +110° C under nitrogen. The aminoacids were separated using a Biocal 200 automatic aminoacid analyzer. The PGs prepared from tissue fractions II and IV sufficed only for these analyses. The further study was therefore performed on PGs from tissue fraction III only.

Materials representing the 45–60 ml, 64 76 ml and 90 100 ml intervals of the Sepharose 6 B eluate from tissue fraction III (cf. Fig. 3 b) were pooled and digested with papain. These fractions

The authors are indebted to Dr *Dick Heinegård* for the aminoacid analysis.

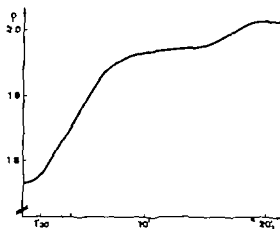


Fig 1 Densities of the gradient used. The tissue powder was fractionated at the plateaus whereby convection factors were minimized.

were designed 6B-2 6B-3 and 6B-4 respectively The void volume of the gel chromatogram was not collected because of the small amount of material there. To determine the amount of glycoprotein bound hexosamines, the digests were chromatographed on Ecteola-cellulose as described above.

To investigate whether or not papain digestion —a procedure known to liberate single GAG chains from PGs (*Scott* 1960)—was able to decrease the molecular size of the isolated material, the PGs from tissue fraction III as well as from the 6B-2 and 6B-3 fractions were digested with papain and GAGs were isolated as described above. The polysaccharides were added to the Sepharose 6 B column previously described, and the hexosamines of the effluents were determined using the method of *Elson & Morgan*.

RESULTS

The gradients proved to remain discontinuous enough to give well defined bands (Fig 1) This finding is also reflected in the low standard deviation of the registrated fraction limits (Table 1)

The Ca, P and N analyses (Table 2) show the differences in degree of mineralization between the tissue fractions. The Ca/P ratios approximate the theoretical value of hydroxy apatite, which is 2.17 w/w (molar ratio of 1 67) The Ca/N and P/N ratios are consistent with the figures given by *Engfeldt & Hjerpe* (1974) The three fractions correspond to the early middle and late part of the final bone mineralization, i.e. nonmineralized osteoid was not isolated. Thus the earliest part of bone mineralization could not be studied.

TABLE 1 *The Density Intervals and Pooled Recovery of the Bone Powder Fractions. The Total Recovery R presents 70 Per Cent of the Powdered Material*

Fraction		Density intervals (g/cm³ ± SD)			Yield (g)
I	(float)			1 785 ± 0.01	0 074
II	(least mineralized)	1 785 ± 0 012	–	1 958 ± 0 007	2.210
III	(medium mineralized)	1.958 ± 0.007	–	2 023 ± 0 006	7.316
IV	(most mineralized)	2.023 ± 0.006			4.024
Total					13.624

TABLE 2 *The Ca P and Amino-N Contents of the Three Studied Bone Tissue Fractions*

Fraction	Amount of material hydrolysed (mg)	Content of Ca (µg/mg)	P (µg/mg)	N (µg/mg)	Ca/N w/w ratio	P N w w ratio	Ca/P w/w ratio (molar ratio)
II	0 932	217	10[illegible]	43.5	4 98	2 35	2.12 (1.64)
III	0.936	235	109	40.9	5.75	[illegible]	[illegible].16 (1.67)
IV	0.882	238	111	38.5	6.18	2 89	2.14 (1 65)

TABLE 3 *DEAE Microcel mn Chromatography of the CPC Supernatant of Papain Digested Material*

Eluant	II		III		IV	
	Hex (µg)	S.A.** (µg)	Hex (µg)	S.A.** (µg)	Hex (µg)	S.A.** (µg)
H_2O + 0.06 M HCl	13.0	10.6	11.9	10.0	9.7	8.2
0.3 M NaCl	–	–	–	–	–	–
6 M HCl	–	–	–	–	–	–

The low ionic strength of the eluant and the large content of sialic acid indicate that the material is of glycoprotein origin.

* Hex = hexosamines.
** S.A. = sialic acid.

Glycoprotein Analyses

DEAE-cellulose chromatography of the supernatants from CPC precipitations of papain digested tissue (Table 3) showed that all the hexosamine containing material was totally eluted with 0.06 M HCl, too low an acid concentration to elute the glycosaminoglycans (*Antonopoulos* personal communication). Therefore it can be assumed that the hexosamines eluted with water or 0.06 M HCl are part of glycoproteins. The high contents of sialic acid in the fractions, with a hexosamine/sialic acid ratio of about 1.2, also seem to indicate that the hexosamines are not of glycosaminoglycan origin, but rather oligosaccharides derived from glycoproteins. The total amounts of glycoprotein-hexosamines in the tissue fractions, as calculated from the results of the DEAE-chromatography (Table 4) do not differ significantly

TABLE 4 *The Glycoprotein Bound and GAG-Bound Hexosamines of the Bone Tissue Fractions Calculated on Organic Dry Weight Basis Assuming that All CaO is in the Hydroxy Apatite Form*

Fraction	Glycoprotein bound hexosamine µg/mg org dry weight	GAG-bound hexosamine µg/mg org. dry weight	Total hexosamine µg/mg org dry weight
II	1.12	0.53	1.65
III	1.09	0.48	1.57
IV	1.24	0.40	1.64

Glycosaminoglycans

The GAG bound hexosamines, correlated to organic dry weight, show a significant decrease from the least to the most mineralized fraction (Table 4). CPC-cellulose chromatography was used to separate GAGs. Only traces of hexosamine were found in the 0.3 M NaCl fraction (Fig 2). The remaining GAGs were only galactosaminoglycans as estimated from ion exchange chromatography of acid hydrolysates. The major part (95 per cent) of the GAGs were eluted with n-propanol/methanol from CPC-cellulose columns and only traces of material were detected in the other fractions, indicating that most of the material is CS-4 (*Antonopoulos & Gardell* 1963). However the material eluted with the 0.3 M NaCl solution might prove to be hyaluronic acid. The amount of material in this fraction was too scarce for further identification. The three tissue fractions were similar regarding CPC-elution patterns. Thus, elution profiles, with increasing ionic strength (*Fig. 2*) show similar patterns as the bulk of the material was eluted with the 0.55 M $MgCl_2$. A slight difference between the tissue fractions was noted: the GAGs of the most mineralized material were eluted somewhat earlier than those of the less mineralized tissue, showing more material in the first $MgCl_2$ fraction even when calculated on organic dry weight basis.

The sulphate contents of the GAGs were similar, the molar sulphate/hexosamine ratios being 1.0, 1.1 and 1.1 for fractions II–IV

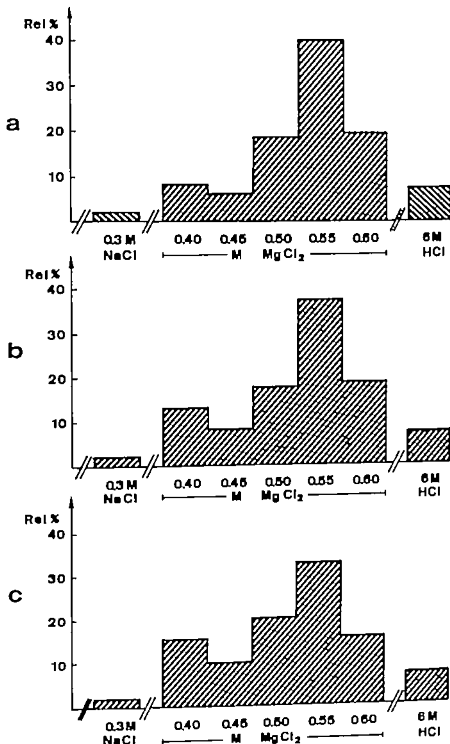


Fig 2 Profile of GAGs from least (a) medium (b) and most (c) mineralised bone tissue eluted from the CPC columns with neutral $MgCl_2$ sol tions with increasing ionic strength. Note the slight shift toward lower electrolyt concentration with higher degree of mineralisation.

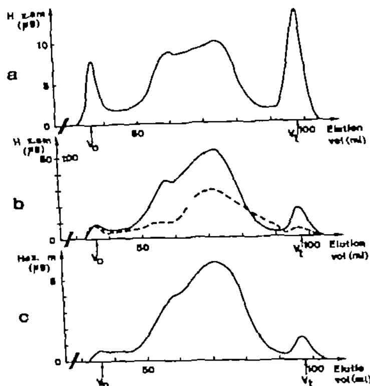


Fig 3 Chromatography on Sepharose 6 B of proteoglycans from least (a) medium (b) and most (c) mineralized bone tissue showing the hexosamine distribution (———) and for the medium mineralized tissue also the protein contents (– – – –)

respectively Therefore the CPC-cellulose profile indicated that the GAGs of most mineralized bone have a somewhat smaller molecular size than those of less mineralized bone.

Proteoglycans

The PGs of the three tissue fractions were extracted with similar yields, and they were eluted from the Sepharose 6 B column in three peaks (Fig. 3) one in the void volume, one major broad biomodal retarded peak and, finally one peak eluted in or close to the total volume. The proportions between the three peaks differ in the chromatograms in that the proportion of the v to the v_t peaks decrease in the most mineralized tissues. When calculated on organic dry weight basis, there is no difference between the tissue fractions in the total amounts of the biomodal intermediate peaks. Therefore most of the observed differences can be explained by decreases in the v and v peaks with increasing degree of mineralization. The proportion of the larger molecular size material in the major biomodal peak, however was different in the three tissue fractions. Thus mineralization was followed by slight relative decrease in material of higher molecular weight, while the material eluted later showed a simultaneous increase The k_a values of these peaks showed only minor differences.

The predominating amino acids of the PG preparations were aspartic acid, serine, glutamic acid, proline and glycine (Table 5) There were differences in the aminoacid composition, but consistent trends were difficult to find. The analyzer was unfortunately slightly overloaded with the sparse material from tissue fraction IV resulting in somewhat uncertain values of aspartic acid, glutamic acid and glycine

For the further analysis material from tissue fraction III only sufficed. Proteins were

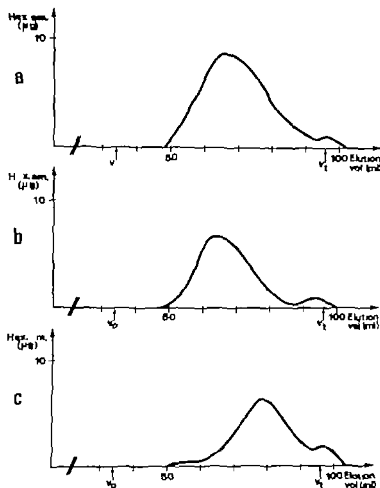


Fig 4 Chromatography on Sepharose 6 B of papain digested proteoglycan preparations from medium mineralized bone tissue (a) and from PG subfractions 6B-2 (b) and 6B-3 (c)

found in all fractions of the Sepharose 6 B chromatogram. The protein/hexosamine ratios, however were different in the different peaks (1 7 for the excluded peak, 0.5 for fraction 6B-2, 1 1 for fraction 6B-3 and 0.6 for fraction 6B-4 Fig 3 b)

After papain digestion of the PG preparation from tissue fraction III (Fig 4 a) the V_0 peak was absent, while the bimodal major peak was eluted only sligthly later No significant change of the V_t peak could be detected Gel chromatography of the digested PG subfractions 6B-2 and 6B-3 (Fig 4 b, c) showed that these materials, too were only slightly more retarded on the gel, compared to original materials. The material from 6B-2 was eluted with its maximum at 64 ml, while for the 6B-3 material the corresponding figure was 78 ml. In the latter chromatogram there was also a small peak in the total volume.

Ecteola cellulose chromatography of the papain digested 6B-2 fraction showed that 10 per cent of the hexosamines were eluted with 0 06 M HCl. For the 6B-3 material this figure was 42 per cent and for the 6B-4 material it was as high as 7? per cent. In all cases the 0.3 M NaCl fraction showed traces of hexosamine The remaining hexosamine was eluted with 6 M HCl, in which fraction CAGs would elute.

DISCUSSION

The results indicate that in all three tissue fractions CS-4 was the major CAG possibly with minor traces of hyaluronic acid, demonstrated in bone tissue *in toto* by other authors (*Rogers* 1949 *Meyer et al* 1956 *Hjertquist*

TABLE 5 *The Aminoacid Composition of the Isolated PGs. The Figures Are Given as Ratio to Leucine*

	Bone tissue fraction		
	II	III	IV
Asp	3.9	2.7	7.4*
Thr	1.7	1.3	2.4
Ser	2.1	1.6	2.5
Glu	4.6	4.1	7.5*
Pro	2.1	5.2	2.1
Gly	2.8	3.2	4.0*
Ala	1.3	1.3	1.6
Cyst	–	–	0.09
Val	0.99	1.1	1.0
Met		–	–
Iso	0.50	0.62	0.49
Leu	(1.0)	(1.0)	(1.0)
Tyr	0.64	0.61	0.69
Phe	0.40	0.47	0.50
Lys	0.67	0.95	0.53
His	0.31	0.27	0.19
Arg	0.66	0.88	0.66

This value may be too low because of high extinction values, due to overloading of the column.

& Vejlens 1966). The total amount of CS seems to decrease during the period when the degree of mineralization increases from low levels to final values. Based on our studies, the present report supports and extends those of *Pugliarello et al* (1970) who studied the total hexosamine contents in osteones of different density. The change in CS quantity is also accompanied by a slight shift toward GAGs of lower average molecular weight in the more mineralized matrix.

Most of the GAGs extracted with guanidinium chloride are probably bound to proteins in the form of PGs as shown by *Herring* (1968). Since the GAGs are eluted at relatively high electrolyte concentrations from the CPC-cellulose columns, the CS chains are rather large (*Antonopoulos et al* 1964). Contrary to this, most of the PGs are partly included in the Sepharose 6 B gel, indicating low molecular weight of this compound compared to cartilage PGs. These facts and the small change in gel chromatographic pattern after papain digestion, however, indicate that each PG molecule carries only a few GAG chains. The protein/hexosamine ratios are high compared to cartilage PGs. These high ratios are, however, similar to those reported by *Herring* (1968) and it is not very likely that they are caused by protein contamination.

To investigate how a contingent autolytic degradation of PGs during storage of the bone could influence the results, control experiments were undertaken. According to the figures given by *Vejlens* (1971) autolytic degradation of the GAGs is not to be expected when the bone is left for 2 days at +4 C. To study possible autolytic effects on bone PGs, the gel chromatographical pattern of PGs extracted from canine bone tissue, has been examined as a separate experiment (unpublished). Prior to PG extraction the tissue representing segments of the same bone, had been kept at +20 C for 2 hours and subsequently at +4 C for varying times. The control material, on the other hand, was extracted directly. No significant change in gel chromatographical behaviour or in the degree of extraction of the PGs could be detected after 2 hours at +20 C and subsequent 5 days at +4° C. When the material was stored at this temperature for 10 days the recovery decreased by 26 per cent and a part of the PGs was more retarded on Sepharose 6 B (Fig 5) possibly due to autolytic effects. Since the human bone was studied after 2 days storage at +4 C, autolytic processes probably did not influence the results. To find out if the preparation procedure caused degradation of the PGs, similar experiments were undertaken, comparing the PGs isolated from a coarse powder (mm-large granules) with those extracted from a finely ground and centrifuged material. Since mineralizing dentine is also being studied in a similar manner (unpublished) teeth from beagle puppies were included in these experiments. It could not be demonstrated that the molecular size of the PGs was influenced by the procedure used to prepare the tissue fractions.

Gel chromatography of the papain digested PG subfractions show that PGs of large mo-

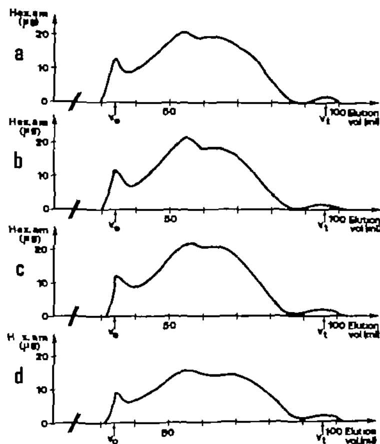


Fig 5 Chromatography on Sepharose 6 B of proteoglycans isolated from dog femur stored at 4 C for 0 days (a) 2 days (b) 5 days (c) and 10 days (d)

lecular size have larger GAG chains than the PGs of smaller molecular size. Thus it seems that the polydispersity of the PGs, at least partly depends upon the polydispersity of its GAGs.

The peaks excluded from the gel chromatograms (Fig 5) represent PGs of considerably larger molecular size. The more intricate nature of this material was not studied in the present investigation.

A large part of the hexosamines in the total volume peak, which peak is more pronounced in the less mineralized tissue, is most likely of glycoprotein origin but GAGs seem to be present. The molecular size of the entities eluted here must be so small however that it is less likely that these CAGs are of PG nature. They might indicate the presence of low molecular GAGs or GAG like oligosaccharides without any connection to PGs, but it is also possible that they are precursors and/or breakdown products in the PG metabolism. The data available do not allow safe conclusions regarding possible variations in GAG contents in this peak.

The aminoacid composition of the PGs is similar to that found by *Herring* (1968). The later part of the mineralization process in bone is accompanied by a change in the aminoacid composition of the PG preparations. As mentioned above the earliest mineralization could not be studied with the present method. Judging from Ecteola-cellulose chromatography the isolated PG material also contains glycoprotein type oligosaccharides as shown by *Herring* (1968). Similarly glycoproteins have been demonstrated in PG preparations from aorta cornea and sclerae (*Antonopoulos et al.* 1974). The glycoprotein contents are larger in the gel fractions of small molecular size and largest in the total volume peak, where they represent a major proportion of hexosamines. Still CAG-bound hexosamines are present also in this fraction,

judging from Ecteola-cellulose chromatography.

As the total amount and average molecular size of CS decrease during the last phase of the mineralization of bone, the calcium binding capacity of these GAGs might decrease and it is possible that the GAGs participate to some extent in the mineralization process by delivering calcium ions, which could enhance nucleation. The amount of calcium ions delivered must, however be very small compared to the amounts of calcium hydroxyapatite deposited during the late phase of bone mineralization. Cartilage PGs are known to influence the mineralization process *in vitro* (*Cuervo et al.* 1973). The importance of the changes in bone PG structure during the mineralization process is, however still obscure. Further studies concerning the calcium binding qualities of bone PGs are necessary before safe conclusions can be drawn in this respect.

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FOETAL RHABDOMYOMA

Case Report of a Patient with Two Tumours

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Dahl, I., Angervall, L. & Säve-Söderbergh, J. Foetal rhabdomyoma. Case report of a patient with two tumours. Acta path. microbiol. scand. Sect. A, 84: 107–112, 1976.

A case report of a girl with two foetal rhabdomyomas is presented. One tumour was a small cutaneous lesion present at birth in the left thigh and the other was located in the chest wall deeply in the subcutaneous tissue and attached to the adjacent intercostal muscle. The patient also had the naevoid basal cell carcinoma syndrome which lends credence to the opinion that foetal rhabdomyoma is a malformation rather than a true neoplasm.

Key words: Rhabdomyoma; soft tissue tumour; naevoid basal cell carcinoma syndrome.

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Nine cases of a distinctive subcutaneous soft tissue tumour composed of myoblasts in different stages of differentiation have been reported from the Armed Forces Institute of Pathology (AFIP) (*Dehner et al.* 1972). The tumour usually became apparent shortly after birth and all patients except one were female. With the exception of one patient who presented two discrete nodules in close proximity, all other patients had solitary tumours and none of the patients in the AFIP-series showed evidence of other tumours or malformations.

This paper presents a clinico-pathological and electron-microscopical study of a patient presenting the naevoid basal cell carcinoma syndrome and two foetal rhabdomyomas, one of which was situated in the corium.

CASE REPORT

A newborn girl presented a small, polypoid cutaneous mass medially on the left thigh. The lesion was excised in September 1967, when the patient was 3 days old. In March 1968, her mother noticed a new mass situated deep in the right side of the chest wall. It was firm, painless, ill-defined, approximately 2 to 3 cm in diameter, located beneath the major pectoralis muscle and fixed to the intercostal muscles. Chest X-ray showed no bone destruction. On April 2nd, 1969, the lesion was excised. According to the surgeon, excision in the neighbourhood of the intercostal muscles was incomplete. At the same time, a small, grey-brown, cutaneous tumour located in the right axillary region was removed. In 1969, the girl presented multiple, small skin tumours resembling nulla, some of which were papillomatous and skin-coloured, others brown. On the right side of the chest, X-ray showed deformed, bifurcating ribs. Examination of the eyes showed peripheral hypoplasia of the iris-stroma in the irido-corneal angle, but there were no signs of glaucoma. The size of the head was slightly increased, but X-ray of the skull revealed no abnormalities. In 1971, approximately 50 small skin lesions were excised; 20 of these were examined histologically. Electro- and echo-encephalography showed normal conditions and the results of psychological test (Griffith) were within normal limits for all functions examined. In 1972, multiple small, dark skin tumours, located in both inguinal regions and supraclavicularly in both axillae, were treated by diathermy. Skull X-ray revealed a small calcification, probably situated in

the falx cerebri. X ray of the teeth and supporting structures of the teeth showed that all permanent teeth except 5+ and +5 were well-developed. Since then, the patient has been under regular observation. She has been progressing normally physically as well as mentally

METHODS

Light Microscopy

The operative specimens were fixed in 10 per cent formalin solution and embedded in paraffin. *Five μm thick sections were routinely stained according to the haematoxylin-van Gieson method* and with haematoxylin and eosin. Gordon's silver impregnation was used for the demonstration of reticulum fibres. Staining with phosphotungstic acid-haematoxylin (PTAH) and Masson's trichrome stain was performed in order to visualizing the cytoplasmic striation. The extracellular ground substance was stained for acid mucopolysaccharides by Alcian blue (Chroma-Gesellschaft) at pH 2.5 and 0.5 and by *toluidine blue at pH 4 and 0.5* with and without preceding treatment of the sections with testicular hyaluronidase (hyaluronidase from bovine testes, type IV Sigma). The periodic acid-Schiff reaction (McManus) was performed with and without preceding treatment of the sections with diastase (Merck, 2800 E/g)

Electron Microscopy

Only paraffin-embedded material was available from the two mesenchymal tumours. Small pieces from selected areas of 6–20 μm thick sections from the paraffin blocks were carefully deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol and finally washed in cacodylate buffer. Thereafter the tissue was fixed in 1 per cent osmium tetroxide in cacodylate buffer pH 7.2 for 2 hours, dehydrated in ethanol and embedded in Epon 812. Ultrathin *sections were cut in an* LKB Ultrotome III stained with uranyl acetate and lead citrate examined and photographed in a Philips EM 200 electron microscope.

RESULTS

Light Microscopy

Almost all the skin tumours examined histologically were *basal* cell carcinomas. Two tumours, one of which was located medially on the left thigh while the other was located laterally on the right side of the chest were mesenchymal tumours situated in the superficial soft tissue the small in the corium (*Fig. 1) and the large* in the subcutaneous tissue adjacent to muscle fascia. Both tumours were ill-defined without encapsulation. The tumour cells were haphazardly arranged in a myxoid ground substance which was most abundant in the centre of the tumours (Fig. 4). The ground substance was not stained by the periodic acid-Schiff reaction, but seemed to contain hyaluronidase-labile acid mucopolysaccharides demonstrated by the Alcian *blue and toluidine blue stains. The staining reactions* with Alcian blue and toluidine blue at the low pH's were negative and thus, no sulphated mucopolysaccharides (glucosaminoglycans) were demonstrated. The tumour cells, varying in diameter were elongated strap- or ribbon-shaped. The nuclei were small, oval or elongated, and *in places* arranged in rows either in the centre or at the *periphery of the cells. Scattered among these cells* were small, round or oval cells with *tapered cytoplasmic* processes. In the small tumour the tumour cells were generally more elongated, the nuclei mostly being arranged centrally (Fig. 2). However in a few broader tumour cells, the nuclei were more often marginal (Fig. 3). In the large tumour broad, cytoplasmic tumour cells with elongated nuclei peripherally often arranged in rows, were frequently observed (Fig. 5). In both tumours, the cytoplasm was picrinophilic it stained brick-red with Masson trichrome stain and contained longitudinal fibrils. Significant cross-striation was demonstrated only in the broad tumour cells found in the large tumour (Fig. 6). Mitoses were not observed in the *tumour cells. Some inflammatory* cells, particularly mast cells and eosinophilic leucocytes, were scattered among the tumour cells.

Electron Microscopy

In spite of the formalin fixation, the overall preservation permitted an ultrastructural analysis which was directed especially at the fibrillar structures in the cytoplasm.

In the large mesenchymal tumour the abundant cytoplasm contained aggregates of filaments arranged in parallel (Fig. 7). It was not possible to differentiate between thick and thin filaments. Cross-banding of the bundles of filaments was evident with formation of conspicuous Z lines and A bands, identifiable I bands, but no certain

Fig. 1 The small tumour *located* the corium and adjacent subcutaneous tissue. The growth of the tumour is diffuse the epidermis irregularly acanthotic. Masson's trichrome × 40

Fig. 2 Elongated strap- or ribbon-shaped tumour cells in the small tumour. The nuclei are elongated, centrally placed and arranged rows. Round or oval tumour cells with tapered cytoplasm mingle with these cells. Masson trichrome 400

Fig. 3 Broad tumour cell in the small tumour. The cytoplasm shows distinct longitudinal fibrils and the nuclei are marginally situated Masson trichrome × 630.

5

6

7



Fig 8. Small tumour. Tumour cell containing wavy filaments mainly arranged in parallel with condensed areas. × 6,800.

M lines or H zones. The aggregates of filaments were irregularly placed in the cytoplasm, but displaying a definit tendency towards parallel arrangement along the longitudinal axis of the cells. Ribosomal particles were seen which in some places were in "Indian-file" formation. Identifiable mitochondria were not seen. Other cytoplasmic details did not reveal any characteristics deemed worthy of interest.

The small tumour (Figs. 8) displayed less characteristic cytoplasmic filaments and significant cross-banding was absent. The cytoplasm of the long slender cells contained slightly wavy filaments mainly arranged in parallel. In some cells, the filaments seemed to congregate and to be surrounded by electron-dense material giving the impression that Z lines were developing especially in some areas where the aggregates of filaments tended to lie in rows. Thin and thick fibres could not be identified.

Fig 4. Haphazardly-arranged, elongated tumour cells of varying diameter in the large tumour. Centrally the myxoid ground substance is abundant. Masson trichrome × 40

Fig 5. Small and broad, elongated tumour cells mingling with round and oval cells with tapered cytoplasm in the large tumour. The myxoid ground substance is focally abundant (upper left) and seems to be vacuolated. Masson trichrome × 100.

Fig 6. Broad tumour cells in the large tumour. The cytoplasm shows longitudinal fibrils and distinct cross striation. PTAH × 630.

Fig 7. Aggregates of filaments arranged in parallel definite Z-lines are seen in the large tumour × 23 400.

DISCUSSION

The two soft tissue tumours exhibited a mixture of elongated, strap- or ribbon-shaped cells as well as primitive small, round or oval cells similar to or identical with the socalled promyoblasts (*Fishman* 1972).

Mature skeletal muscle fibres with fully established cross striation and marginal nuclei

were demonstrated only in the large tumour. The microscopic appearance of this tumour is similar to that of foetal rhabdomyoma in subcutaneous tissue described by *Dehner et al* (1972).

In the small tumour which was situated in the corium, the elongated tumour cells were generally more narrow with a central nucleus. These cells resemble the so-called myoblasts, i.e. the cells present in the early stages of the development of skeletal muscle fibres (*Fishman 1972*). Significant cross-banding or cross striation could not be demonstrated either by light or by electron microscopy. However the cytoplasmic structure, where fibrils were arranged in parallel and a suggestion of Z line formation was noted, as well as the position of the nuclei seem to justify the classification of this tumour as a foetal rhabdomyoma.

The rhabdomyomatous tumours in the present case did not show any cellular and/or nuclear atypia. Hyperchromatic or large, bizarre nuclei, mitoses or areas of necrosis were not seen. These findings, combined with the innocent clinical behaviour indicate that the tumours were benign. As pointed out by *Dehner et al.* (1972) however the distinction between foetal rhabdomyoma and well differentiated embryonal rhabdomyosarcoma may be subtle, clinically as well as histologically. Differentiation between these two tumour types may be rendered more difficult as they occur in a similar age group and their anatomical distribution is similar. However the auricular area, which was the most common site of foetal rhabdomyoma in the AFIP-series, seems to be an exceedingly rare site of embryonal rhabdomyosarcoma (*Angervall et al.* 1972). We agree with *Dehner et al* (1972) in that foetal rhabdomyoma histologically can be clearly distinguished from adult rhabdomyoma.

Dehner et al (1972) expressed their scepticism concerning the accuracy of the term "foetal rhabdomyoma" since the precise nature and origin of this tumour remain unknown. They were of the opinion that the lesion may be a hamartoma and not a true neoplasm since the lesions exhibited the presence of myoblasts in early stages of differentiation. In contrast to the patients described by *D hner et al.* (1972) our patient also presented multiple basal cell carcinomas associated with anomalies of ribs and the iridocorneal angle of the eye. Thus, the disease in our patient fulfils the criteria of the naevoid basal cell carcinoma syndrome (*Gorlin et al.* 1965 *Mason et al.* 1965) which has been included in the group of phacomatoses (*Hermans et al.* 1965). The appearance of the foetal rhabdomyomas in such a patient lends credence to the opinion that foetal rhabdomyomas are malformations rather than true neoplasms.

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EFFECTS OF VIRAL AND MYCOPLASMA INFECTIONS ON ULTRASTRUCTURE AND ENZYME ACTIVITIES IN HUMAN SKELETAL MUSCLE

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Aström, E., Friman, G. & Pilström, L. Effects of viral and mycoplasma infections on ultrastructure and enzyme activities in human skeletal muscle. Acta path. microbiol. scand. Sect. A, 84 113–122, 1976

The effect on skeletal muscle of acute viral and mycoplasma infections in thirteen men of ages ranging between 20–42 years has been studied. Comparisons are made with eight healthy men in the age group 22–29 years who were confined to bed for periods of time of lengths similar to the confinement to bed of the patients. Muscle samples were taken from the thigh. Glyceraldehyde 3-phosphate (triosephosphate) dehydrogenase (TPD) lactate dehydrogenase (LDH) citrate synthetase (CS) and cytochrome oxidase (cytox) activities were measured and the ultrastructure of the muscle specimens was studied by electron microscopy Immobilization of the healthy persons induced decreased activities of CS but those of TPD LDH and cytox remained unaffected Return to normal life restored the CS activity The activities of the four enzymes were lower in the patients than in the healthy subjects after immobilization. During normal life the activities slowly rose to levels as those seen in the healthy subjects. In connection with the acute disease focal ultrastructural changes within the muscle were found. The changes were similar to those reported to occur in other more specific muscle diseases.

Key words: Mycoplasma pneumonia viral infection skeletal muscle ultrastructure enzyme activity

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In the clinical routine, it is not uncommon that patients suffering from acute infections complain of sore or aching muscles and of reduced physical fitness. The pathogenesis of these symptoms is not fully understood. Reports are available according to which virus-like particles have been observed in the muscle fibres in patients suffering from chronic polymyositis (6, 21) and in two cases of complicating myositis involved in herpes zoster (20) However similar phenomena have been observed in patients with muscular dystrophy (17) thus suggesting that these particles, whatever their nature, may be secondary to the disease. It is not until recently that it has been possible to isolate virus from muscle tissue which by electron mi

Technical assistance by Anna-Stina Sten ...

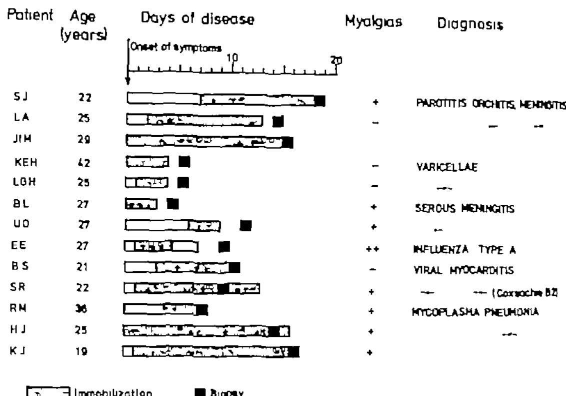


Fig 1 The group of patients examined. Age, number of days of clinical illness, immobilization and biopsy occasion. — + and + + denote absence of symptoms, moderate, and severe symptoms of myalgias, respectively

croscopy was shown to contain picornavirus-like particles, i.e. in a case of chronic myopathy (30)

Enzymatic alterations are reported to occur in muscle tissue in connection with different muscular diseases producing distinct symptoms (24) However symptoms of pain need not be indicative of a presence of enzymatic alterations. In a group of alcoholic patients without muscular symptoms of fatigue or pain the activities of lactate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase were lower than those in a group of untrained men of similar ages but without any known abuse of ethanol (14)

The present investigation is a controlled study of the ultrastructure and enzymatic activities in skeletal muscle biopsies from patients during and after viral and mycoplasma infections.

MATERIAL AND METHODS

Subjects

Thirteen male patients aged 20–42 years who were hospitalized at the Department of Infectious Diseases, University Hospital in Uppsala, were selected to participate in the investigation. Ten had different viral diseases and three had mycoplasma pneumonia (Fig 1) They were included in the group for study with no further criteria for the selection other than age and sex. All willingly gave their permission to let biopsies be taken. One patient (K.E.M. in Fig. 1) had a mild bronchial asthma but had not received cortisone medication. Chronic diseases were not manifest in the other patients

Eight healthy men aged 22–29 years served as a control group. They were immobilized in bed for seven days, one at a time, in a special room on the ward. They followed the same daily routine as the patients, the aim being to achieve a degree of physical activity and caloric intake of the same order as that in patients.

Clinical Procedure and Findings

All patients were examined by one of the authors. As regards admission of patients, the routine procedure was followed in all cases of acute in-

fectious disease, including record of the past medical history complete physical examination, haemoglobin assay erythrocyte sedimentation rate white blood cell count (WBC) differential count and urinanalysis for albumin, glucose and cells A serum sample was drawn and stored at −21 C for later serology With a view to confirming the diagnosis, lumbar puncture was performed in the cases of meningitis, an electrocardiogram was obtained if myocarditis was in evidence and a chest X-ray in the presence of pneumonia Acetyl salicylic acid was given to reduce fever and myalgias, if present. In cases of pneumonia, erythromycin was given in addition.

The illness was considered to start at the time when the first symptom occurred and to be over when the rectal temperature was less than 37.5 in the morning or 38 0 in the afternoon. In one case of myocarditis (B.B. in Fig. 1) the absence of fever prevented the use of the latter standard and the first day out of bed was used instead. The duration of the illnesses averaged 10.0 ± 1.5 days, the duration of the fever averaging 8.0 ± 1.6 days and that of immobilization 8 6 ± 1 4 days. Clinical examination did not in any case reveal unexpected complications to the illness concerned.

The aetiological diagnosis could be established serologically in paired sera in all but three of the patients (i.e. B L., U Ö. and B.S. Fig. 1) A four fold titre rise was required, but in two cases (S.J and J.J.S.L.) a high titre was registered in both sera and accepted as diagnostic. (The first sample of serum was obtained two weeks after onset of the illness.) Tests of complement-fixing (CF) antibodies were performed in all cases (Department of Virology University of Uppsala) In addition, a disc neutralization test containing a great number of different enteroviruses (M. Lagercrantz, Statens Bakteriologiska Laboratorium, Stockholm) was used in four patients (B.L., U Ö B.S. and S.R.) A rise in titre of Coxsackie B2 antibodies was registered in one of these cases (S.R.) while no aetiological agent could be observed in the others. Tests of CF antibodies of mycoplasma pneumoniae, parotitis, herpes simplex, and RSSE-agents were negative. No viruses could be isolated from the faeces.

Biopsy

Biopsies were taken percutaneously from m. vastus lateralis by the needle technique described by *Bergström* (4) after local anaesthesia with 5.0 ml lidocain subcutaneously in the biopsy area. Anaesthetization of the fascia and muscle was avoided. Each successive biopsy was taken 2 cm proximal to the preceding one in the same leg. All biopsies were taken by the same investigator The sample was divided into two parts—one for assay of enzyme activities and one for electron microscopy

In the patient group a muscle biopsy was taken in direct connection with the acute disease (12.1 ± 1.5 days after the onset of the disease) and follow-up biopsies after 49.9 ± 9 0 days and 128.5 ± 6 6 days, respectively In the control group, an initial biopsy was taken immediately before the trial (day 1) a second at the end of the bed rest period (day 8) and follow-up biopsies after 41 4 ± 3.3 days and 110.9 ± 11 7 days, respectively

Enzyme Assay

The biopsy was rapidly weighed and homogenized in twenty times (weight to volume) ice-cold 0 15 mol/l KCl 6 mmol/l EDTA, 50 mmol/l $KHCO_3$, pH 7 4 using a Potter-Elvehjelm all-glass homogenizer The homogenization was performed by hand six times for thirty seconds with intervals of one minute between procedures to avoid a rise in temperature. The whole procedure was performed at 0 –4 C. Using this homogenate, the activities of citrate synthetase (CS E.C.4 1.3 7) glyceraldehyde-3-phosphate dehydrogenase (triose phosphate dehydrogenase (TPD E.C.1.2 1 12) lactate dehydrogenase (LDH E.C.1 1 1.27) and cytochrome oxidase (cytox E.C.1.9.3 1) were estimated. The method used for CS was that described by *Srere* (28) The methods used for TPD and LDH were those described by *Bass et al.* (3) and for cytox, the method described by *Tottmar et al.* (31) was used.

Electron Microscopy

Part of the biopsy was rapidly transferred to ice-cold 2 per cent OsO_4, according to Rhodin (23) and cut by a razor blade into pieces smaller than 1 mm The fixation continued in the same fixative for two hours at 0–4 C. The specimens were washed in Tyrode's solution for 30 min and then dehydrated in ethanol and embedded in Epon 812. Sections were cut by glass-knives using a LKB-ultramicrotome, stained with uranyl acetate and basic lead citrate and studied in a Siemens Elmiskop, type 101

RESULTS

Enzymes

Immobilization of the healthy subjects for one week induced a significant ($p < 0.05$) decrease in the activity of citrate synthetase (CS) and a greater although non-significant, decrease in lactate dehydrogenase (LDH) activity in skeletal muscle. The activities of

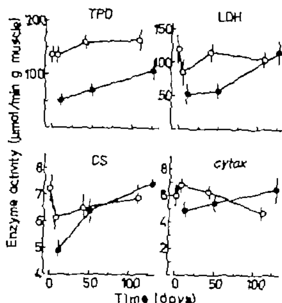


Fig 2 Activities of the four enzymes glyceraldehyde 3-phosphate dehydrogenase (TPD) lactate dehydrogenase (LDH) citrate synthetase (CS) and cytochrome *c* oxidase (cytox) in skeletal muscle biopsies from thirteen male patients (filled circles) who just before the first biopsy was obtained were confined to bed because of different viral and mycoplasma infections but afterwards were living a normal life. Open circles denote the same activities in eight healthy controls of the same age and sex who were staying in bed for one week (days 1–8) and thereafter returned to normal life. The circles denote mean values the bars denoting the S E.M.

triosephosphate dehydrogenase (TPD) and cytochrome oxidase (cytox) remained unaffected by immobilization. One month after the return to normal life, the activities of these four enzymes were on the preimmobilization levels (Fig 2)

In direct connection with the acute disease, the activities of the four enzymes were lower ($p < 0.05$ in CS LDH and cytox, $p < 0.001$ in TPD) in skeletal muscle of the patients than in the muscle of the healthy control subjects after one week's immobilization (Fig 2) One month after the return to normal life, the biopsies of the patients still showed a significant decrease in the activities of LDH and TPD as compared with the biopsies of the controls ($p < 0.005$ and $p < 0.001$ respectively) After three months of "normal life" a significant reduction in the TPD activity ($p < 0.01$) still persisted, while the other enzyme activities were on the same level as those in the biopsies of the healthy men at the same time (Fig. 2)

Electron Microscopy

In the group of healthy men no alteration in the ultrastructure of the skeletal muscle could be observed (Fig 3)

In the group of patients with viral and mycoplasma infectious diseases, focal deviations from normal ultrastructure of the muscle were demonstrable in the first biopsy These changes most often affected the organization of the myofibrils and seemed to be of degenerative character The myofibrils were reduced in diameter and a relative increase of sarcoplasm (Fig 4) The Z-lines frequently exhibited structural changes and became irregular in shape and size (Fig 5) sometimes forming dense rods (Fig 6) Many ultrastructural changes were observed in mitochondria some were swollen and possessed less electron dense partly fragmented cristae (Fig 7) In two cases very electron dense inclusions were found within the mitochondria (Fig. 8 a) the origin of these inclusions is obscure. They were probably not ordinary matrix granules because their diameter was 50–80 nm and sometimes they were of a membrane-like appearance (Fig. 8 b)

The amount of glycogen was often increased (Fig 9) and the glycogen occasionally appeared enclosed within membrane bound

Fig 3 Normal ultrastructure of skeletal muscle from a healthy subject after one week in bed Magnification 10000 ×

Fig 4 Degeneration of myofilaments in a patient (E.E.) suffering from influenza type A Magnification 12000 ×

Fig 5 Disorganization of Z-band and myofilaments in a patient (S.J.) suffering from parotitis, orchitis and meningitis. Magnification 16000 ×

Fig 6 Focal disorganization of Z-bands with so-called rod formation in a patient (R.M.) suffering from mycoplasma pneumonia. Magnification 32000 ×















Fig 7 Swollen and partly extracted mitochondrion lacking electron dense matrix and with damaged cristae from skeletal muscle of a patient (B.S.) suffering from viral myocarditis. Magnification 80000 ×

Fig 8 Electron dense inclusions in mitochondria found in two of the patients st died—one (B.S.) suffering from viral myocarditis and the other (R.M.) from mycoplasma pneumonia. Note the membraneous appearance of the inclusions in Fig. 8 b. Magnification a) 32000 × b) 75000 ×

bodies (Fig 9 insert) The membrane seemed to be a double membrane indicating that these glycogen containing bodies could be mitochondria. The sarcoplasmic reticulum was often distended (Fig 10) and it was not always possible to differentiate the sarcoplasmic vacuoles from lysosomal or autophagic vacuoles.

In the second biopsies of the patients only few of these alterations were found and no changes of the ultrastructure were observed in the last biopsies.

DISCUSSION

Enzyme Activities

In enzymatic studies of muscle disease one or two key enzymes have mostly been take



Fig 9 Increased amounts of glycogen granules in a patient (B.L.) suffering from serous meningitis. Magnification: 18000 × Insert: Glycogen containing vacuole in patient (H.J) suffering from mycoplasma pneumonia. Magnification: 40000 ×

Fig 10 Distended sarcoplasmic reticulum—some of the vacuoles might be lysosomes or autophagic vacuoles—in a patient (B.R.) suffering from viral myocarditis. Magnification: 28000 ×

into consideration the lacking activities of which could explain the symptoms, e.g. the activities of the phosphorylases and α-1 4-glucosidase in glycogenosis types V and II, respectively (11 26) In progressive muscular dystrophy (mainly the Duchenne type) the enzymes of glycolysis localized in the sarcoplasm are more affected than those of the mitochondria, many of which are hardly influenced (7 25 32) Thus it would appear *that the enzyme activities of skeletal muscle* during infectious disease follow a similar pattern—reduced activities of TPD CS and LDH but not of cytochrome *c* oxidase (Fig 2) In studies of this type, the inter-individual variation of muscle enzyme activities is great which might be explained by the different fibre composition of the muscle in different individuals (13) In addition, a variable fibre composition exists within the quadriceps muscle in which the percentage of slow twitch fibres is increased in the deeper parts (13) However using a standardized biopsy technique, this error is probably of minor importance (10)

Electron Microscopy

Muscle biopsies obtained in numerous muscle disorders have been investigated by electron microscopy (For review see 18, 22) Usually the alterations occurring in the *muscle cannot be related to specific clinical* conditions although, in a few instances, the changes tend to present a pattern characteristic of a particular disorder It seems as if the muscle responds by a set of several ultra structural alterations. Loss of myofilaments and disruption of the normal banding pattern are the most common abnormalities reported and are unspecific for any particular disease. Hence, it is not surprising to find such alterations *in connection with infectious diseases* (Figs. 4–10) The changes tend to occupy

only small areas of the individual fibres but seem to be widely scattered *focal* areas were found in several microscopic fields within a single muscle sample taken from the largest muscle in the body In a preliminary study (1) a normal structure was observed by light microscopy

The fact that no alterations were found in the group of healthy subjects after one week's bed rest is a little perplexing because disuse atrophy is expected to be the result of this treatment (15) and is also suggested by the fall in CS-activity Muscle glycogen is normally found in the form of single densely osmophilic particles between the myofibrils as well as within the fibrils. When muscle tissue undergoes pathological changes, an increase in the amount of glycogen particles is frequently found (9 19 27) The excess is most commonly accumulated between the myofibrils and beneath the sarcolemma. According to the available reports, this pattern has been seen in many different, unrelated diseases (15 18, 22) and was also found in the present trial (Fig 9) Glycogen particles are sometimes incorporated in membrane limited sacs and are considered to be lysosomes (13 17) However the glycogen containing bodies found in two patients with infections demonstrate a double membrane (Fig 9), indicating that they might be altered mitochondria. This bizarre form of glycogen accumulation within mitochondria has been reported to occur in several neuro-muscular disorders (17 22) The other type of inclusions in mitochondria (as demonstrated in Fig 8) is more difficult to interpret. Various membranous or para crystalline inclusions have been observed in different myopathological conditions, but these inclusions are generally of another size and appearance than those described here (8, 18) *Barnard & Lindberg* (2) have described that different types of mitochondrial inclusions may be present in brown fat cells of rat. They define their type II granule as diameter 40 nm, maximally about 200 nm approximately spherical, electron dense inclusions, frequently showing an obvious internal structure Thus it seems as if the inclusions seen in Fig 8 are very similar to the type II described by *Barnard & Lindberg* It has also been reported that this type of inclusion may be seen in a variety of tissues in animals subject to different pathological or experimental conditions such as dietary deficiency (29) alloxan-diabetes (33) cancer (12) and viral infections (5) *Bienz Isler et al.* (5) have studied the myositis following Coxsackie A infection of suckling mice and found similar inclusions in mitochondria which they consider to be calcium inclusions. This seems to us somewhat improbable because the inclusions may be of a membranous appearance. This problem will be subjected to further analysis. The main results obtained in the experimental study by *Bienz Isler et al* (5) are alteration of the nucleus and increase in number of lysosomal vacuoles. In our study the nucleus seems to remain unaffected. However the size and numbers of vacuoles were rather often found to be increased (Fig. 10) and the origin and function of these must be studied further The findings of swollen mitochondria with less electron-dense matrix and cristae might have been the result of the method of fixation since this type of alteration was not found if another fixative had been used (1) In conclusion in infectious diseases (viral and mycoplasmatic) the skeletal muscle shows enzymatic and ultrastructural alterations which correspond to those found in many other types of muscle diseases, although the extent of the alterations is smaller No direct correlation to the presence of myalgias was found.

We are greatly indebted to the heads of the departments and institute from which this work has emanated, i.e. Prof *Kerstin Landahl-Kiessling*, Dr *Gösta Öberg* and Prof *Gunnar Ström*. Our thanks are also due to Assoc. Prof *Björn Afzelius* *Karl-Heinz Kiessling* and *F Åke Nordberg* for valuable support.

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ACID ACTIVATION OF RENIN IN RABBIT UTERUS

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Jørgensen, J Acid activation of renin in rabbit uterus. Acta path. microbiol. scand. Sect. A, 84 123–129 1976.

During the first 2 days post-partum there is a rapid fall in the uterine renin content of the rabbit. A similar loss of renin can be reproduced *in vitro* when pregnant and post-partum and to a lesser degree non-pregnant uterine tissue slices are incubated at pH 7 4 at 37 C. When a secondary incubation of these previously incubated preparations is performed at pH 4.8 an activ tion of renin is seen. There was not an inverse correlation between the amounts of renin lost during the primary incubation at pH 7 4 and the amounts of renin activated during the secondary incubation at pH 4.8. In similarly incubated preparations of kidney cortex, where only small per cent of the initial renin content is lost during the primary incubation at pH 7 4 no activatable renin is seen.

Key words Renin acid activation rabbit uterus.

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In two previous papers it was shown that the renin in non-pregnant, pregnant and post partum rabbit uterus (*Jørgensen* 1974 1976) was partly inactivated when they were incubated at pH 7 4 *in vitro* In similar incubations of slices of kidney cortex a smaller relative decrease in renin was seen. Contrary to these effects of incubation at pH 7 4 it has been shown that incubation at an acid pH reveals an activatable renin in different human and mammalian tissues human amniotic fluid and plasma (*Lumbers* 1971 *Morris & Lumbers* 1972, *Skinner et al.* 1975) Wilms tumor of the human kidney (*Day & Luetscher* 1974) hog kidney (*Boyd* 1972 and 1974 *Rubin* 1972) and rabbit kidney (*Leckie* 1973 and 1975)

In the present paper the existence of activatable renin in rabbit uterus is demonstrated (part I II) In order to investigate whether this activatable renin is a pro-enzyme of renin or an intermediary product of the above mentioned renin inactivation, slices of non-pregnant, pregnant and post partum uterus have first been incubated at pH 7 4 which gives a renin inactivation After this primary incubation a second incubation at lower pH where activation of renin occurs, was performed. For comparison studies of similarly incubated preparations of kidney cortex have been performed (part III)

Furthermore, the distribution of activatable renin between tissue and incubation medium was studied (part IV) Finally the stability of renin and activatable renin during incubation at pH 7 4 has been investigated (part V)

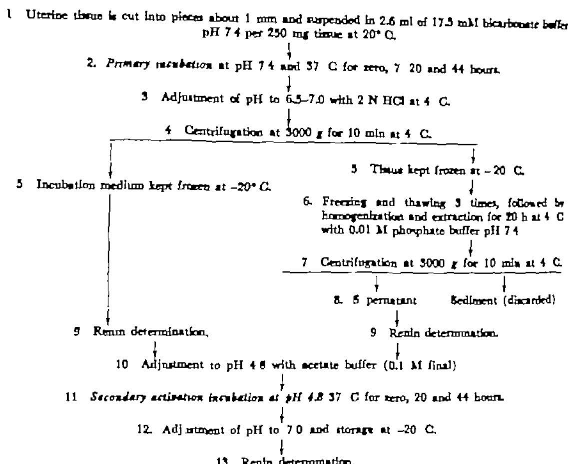


Fig 1 Activation proced e for renin.

MATERIAL AND METHODS

The tissue preparations studied in this paper have been employed in the previous paper (1976)

Animals

Uterine wall and in some cases renal cortex from 8 non-pregnant, 2 pregnant (26th and 27th day of pregnancy) and 2 post partum (about 12 and 22 hours after delivery) albino country rabbits from the State Serum Institute were used.

The primary in vitro *incubation at pH 7 4 and 37° C* was performed as previously described (1974 1976) with quadruplicate incubations for 0 7 20 and 44 hours, pooling of the 4 samples of each incubation period and separation into incubation *medium* and *tissue* after a centrifugation at 3000 *g* for 10 min at 4 C (Fig 1) In the case of non-pregnant animals, uterine tissue from 2 animals was pooled in order to get enough material The tissue was homogenized in phosphate buffer 0.01 M pH 7 4 as previously described (1976)

Activation of Renin (by a Secondary Incubation at pH 4.8 and 37° C)

One ml of incubation medium or supernatant of tissue homogenates was adjusted to pH 4 8 after addition of 0 1 ml 1 1 M sod m acetate buffer pH 4.8. Then 100 µl aliquot were incubated in duplicate for zero, 20 and 44 hours at 37° C (Fig 1) After incubation 1000 µl of 0.2 M Tris/HCl buffer pH 7 5 containing 0 3 per cent human serum albumin was added to ach sample to increase pH to about 7.0 before st rage at –20 C. The renin content before and after incubation at pH 4.8 was measured and calc l ted as previously described (1976) The average of the alues obtained after *20 and 44 hours inc bation* at pH 4.8 wa used since a plateau was reached after 20 hours of incubation.

A control of the renin a t vat n from tissue sediments from homogenization of previously incubated and non-incubated uterine and kidney tissue were frozen and thawed f rther 3 times and rehomogenized in a tissue-buffer ratio of 1-4 The

renin content never exceeded 17 per cent of the original homogenate (n = 12) These homogenates were then incubated for 44 hours at pH 4 8. This incubation gave no significant increase in the renin content.

Renin radioimmunoassay was performed as previously described (*Poulsen & Jørgensen* 1974)

Statistical Evaluation

The intra-assay precision for a single determination in the renin assay was ± 4 per cent (SD) (*Poulsen & Jørgensen* 1974) while the interassay precision was 7 per cent. Differences between experimental values were always determined within the same assay using duplicate and quadruplicate determinations. For convenience the SD was set to ± 5 per cent SD although it was often lower

RESULTS

Part I Time Course of the Activation of Uterine Renin at Low pH

When homogenates and extracts from uterine tissue are subjected to an incubation at pH 4.8 an increase in enzymatic activity of renin is seen. This increase occurs rather slowly (Fig. 2) a plateau being reached after about 16 hours of incubation.

The activated renin has been identified by 1) neutralization of enzymatical activity after addition of antirenin, and 2) the formation of angiotensin I with time after addition of rat plasma at pH 7 4 measured by radioimmunoassay using a specific antibody to angiotensin I (Further data on the characterization of activated renin will be reported in a following paper)

Part II Amounts of Activatable Renin in not Previously Incubated Tissue Slices

a) *Uterus*

In the 4 preparations of *non pregnant uterus* an increase in renin upon incubation at pH 4.8 of 0.3 to 0 6 G U per g tissue is seen (Fig 3 zero hours of incubation, difference between white and black columns) This gives a *relative* increase in renin in all 4 experiments of between 20 and 40 per cent, the initial renin concentrations being from 1 to 3 G U per g tissue.

In the 2 preparations of *pregnant uterus* the amounts of activatable renin are higher about 5 and 4 G U per g of tissue (Fig. 4 A, zero hours of incubation) As the initial renin concentrations are high, 16 and 20 G U per g tissue, the *relative* increase is about the same, 20 and 35 per cent, as in the non-pregnant uterus.

When *post partum uterus* is used an increase in renin of 5 and 1.5 G U per g of tissue is seen upon the incubation at pH 4.8 (Fig 4 B, zero hours of incubation) This *relative* increase is as in the other cases about 30 per cent, the initial renin concentrations being 15 and 5 G U per g of tissue, respectively

b) *Kidney from Non-Pregnant Pregnant and Post-Partum Animals*

When similar incubations at low pH of kidney cortex are performed, no significant

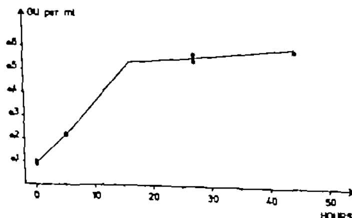


Fig 2 Time course of the activation of uterine renin. Incubation medium from tissue slices of post-partum uterus which had previously been incubated for 20 hours at pH 7 4 37 C, was subjected to secondary incubation at pH 4 8, 37 C after separation from the tissue slices

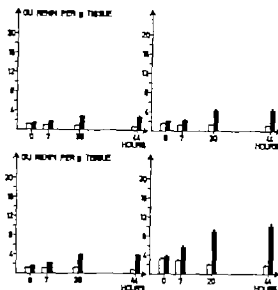


Fig 3 Renin content in non-pregnant uterus with time of primary incubation at pH 7 4 The white columns give the directly measurable amounts of renin in tissue slices plus incubation medium after different periods of incubation at pH 7 4 37 C. The black columns indicate these directly measurable amounts plus the increase in renin measured after a secondary incubation at pH 4.8, 37 C. The bars indicate 1 S D Asterix show statistical significance ($p < 0.01$) of the difference between white and black columns.

alterations in enzymatic activity of renin occur (Fig 5 zero hours of incubation)

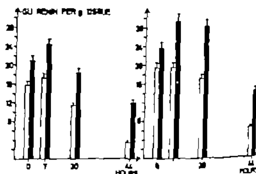
Part III Changes in Acid Activatable Renin in Tissue Slices plus Incubation Medium during the Primary Incubation at pH 7 4

a) *Uterus*

During the primary incubation at pH 7 4 of *non pregnant uterus* the amounts of renin, which are activated by a secondary incubation at pH 4 8, increase from about 0.5 G U per g of tissue initially to from 3–8 G U per g after 44 hours of incubation at pH 7 4 (difference between white and black columns, Fig 3) Although there is a gradual fall in directly measurable renin of about 40 per cent of the initial concentration of from 1–3 G U per g of tissue during the primary incubation (white columns, Fig 3) the sum of directly measurable renin and activatable renin (black columns) is doubled during the primary 44 hours' incubation at pH 7 4

Using *pregnant uterus* the amounts of activatable renin are rather constant, about 8 G U per g of tissue during the 44 hours of primary incubation at pH 7 4 during the last part of which there is a loss of about 70 per cent of the initial concentration of about 18 G U per g of directly measurable renin (Fig 4 A)

In the 2 experiments with uterus taken shortly *after parturition* the amounts of activatable renin in one case were found to be rather constant, about 5 G U per g of tissue independent of the time of primary incubation. In the other case the amounts of activatable renin were about 1.5 G U per g at zero and 7 hours of primary incubation, rising to about 2.8 G U per g after 20 and 44 hours



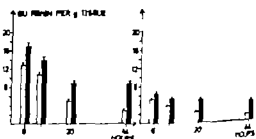


Fig 4 Renin content with time of primary incubation at pH 7 4 in (A) pregnant and (B) post partum uterus.
Symbols as in Fig. 3 Uteri were removed on the 26th and 27th day of pregnancy and about 1 and 22 hours after delivery

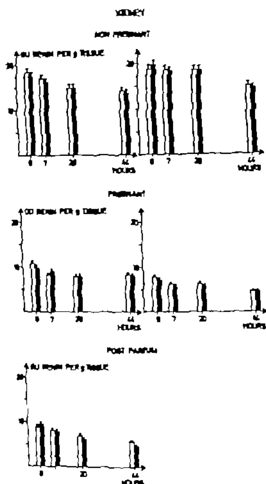


Fig 5 Renin content with time of primary incubation at pH 7 4 in kidneys removed from non-pregnant pregnant and post partum rabbits.
Symbols as in Fig 3. Kidneys were removed from non-pregnant animals, on the 26th or 7th day of pregnancy or about 12 hours after parturition.

incubation (Fig 4 B) During the same incubation about 70 per cent of the initial 15 and 5 G U per g of directly measurable renin was lost. Thus, there is a fall of about 40 per cent of the initial content of directly measurable plus activatable renin (black columns) during the primary incubation.

b) *Kidneys*

Activatable renin was not demonstrated in any of these similarly incubated preparations (Fig 5)

Part IV Distribution of Acid Activatable Renin between Slices and Incubation Medium during the Primary Incubation at pH 7 4

Using *non pregnant* uterus, initially about 20 per cent of the activatable renin is found in the *incubation medium* increasing after 44 hours incubation to about 90 per cent with most of this increase taking place during the first half of the incubation at pH 7 4 Thus, most of the increasing amounts of activatable renin are found in the medium of incubation.

The percentage the activatable renin forms of the directly measurable amounts during incubation, rises in the *tissue slices* from about 30 per cent initially to about 100 per cent after 44 hours' incubation (white columns, Fig 6) at pH 7 4 while in the *medium of incubation* from about 40 per cent initially to *from 450–1150 per cent after 44 hours' in*cubation (hatched columns, Fig 6)

Using *pregnant and post partum uterus* from 20 to 40 per cent of the activatable

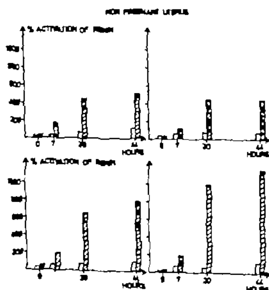


Fig 6 Comparison between the relative increase in renin content in 1) slice and 2) incubation medium from non-pregnant uterus upon the secondary incubation at pH 4.8 with time of primary incubation at pH 7 4
The white columns indicate tissue slices, the hatched columns indicate incubation medium.

renin is found in the *incubation medium* initially increasing to about 70 per cent after 44 hours incubation, with most of the increase taking place during the first 7 hours of incubation Thus, since the amount is constant (see above) the amounts of activatable renin fall in the tissue slices and increase in the medium of incubation.

The percentage, the activatable renin forms of the directly measurable amounts, rises in the *tissue slices* from about 25 per cent initially up to from 40 to 140 per cent after 44 hours' incubation (white columns, Fig 7) In the *medium of incubation* the increase is from 60 to 120 per cent initially up to from 300 to 700 per cent after 44 hours incubation (hatched columns, Fig 7)

Part V Stability of Renin and Activatable Renin during Incubation at pH 7 4

Incubation medium which has been removed from the tissue slices by a centrifugation at 3000 *g* for 10 min, immediately after they have been mixed (zero hours of incubation) contains both directly measurable and activatable renin.

When such samples of incubation media are incubated for 20 hours at pH 7 4 no significant alterations in the content of renin ($p>0.2$, $n = 6$ students t test) and activatable renin ($p>0.2$, $n = 6$) occur

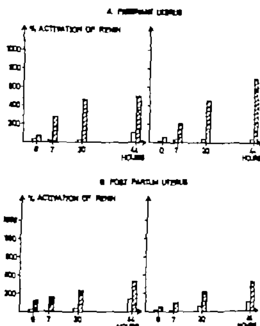


Fig 7 Comparison between the relative increase in renin content in 1) slices and 2) incubation medium from (A) pregnant and (B) post partum uteri upon the secondary incubation at pH 4.8 with time of primary incubation at pH 7 4

Symbols as in Fig 6. Uteri were removed on the 26th or 27th day of pregnancy or about 12 and 22 hours post-partum.

DISCUSSION

The present study has confirmed that the renin content of uterine tissue slices *decreases* with time when the slices are incubated at pH 7 4 It has further been shown that the renin content of such previously at pH 7 4 incubated tissue *increases* after a second incubation at pH 4.8. These two findings could mean that the acid activatable renin was the renin lost at the primary incubation at pH 7 4 If this was the case the inactivation of renin should be due to a conversion of the renin into an inactive form, which then could be reactivated when incubated at low pH. An inverse correlation between the degree of inactivation at pH 7 4 and the amounts of acid activatable renin would then be expected. That this, however was not the case (see the variations in renin and acid activatable renin during incubation in Fig 3 and 4) makes other explanations of the mechanism of the acid activation more probable unless complex relations between de novo synthesis and inactivation occur during the different states of pregnancy Another explanation would be, the presence of an acid activatable prohormone of renin in uterus the amounts of which could increase during the incubation at pH 7 4

In preparations of kidney cortex activatable renin occurred neither before nor during incubation of the tissue This is not in accordance with *Lecktes* (1973 finding of renin activation at pH 2.5 in kidney cortex of New Zealand rabbits using direct bioassay of renin.

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THE EXTENT OF CARCINOMA *IN SITU* IN URINARY BLADDERS WITH PRIMARY CARCINOMAS

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Starklint, H., Jensen, N. K. & Thybo, E. The extent of carcinoma *in situ* in urinary bladders with primary carcinomas. Acta path. microbiol. scand. Sect. A, 84 130–136 1976.

The occurrence of carcinoma *in situ* was examined in a consecutive series of cystectomy specimens from 43 patients. All patients were suffering from or had been suffering from primary bladder cancer. Carcinoma *in situ* was defined as a definitive polymorphia of enlarged nuclei with abnormal chromatin structure in non-tumour bearing areas. It was found in 6 bladders. By means of a systematic technique of cutting the specimens, the extent of the alterations was quantified, relating the number of blocks containing the alteration to the total number of blocks in which the changes possibly might be present. The extent of carcinoma *in situ* ranged from 2 to 81 per cent with an average of 20 per cent. The distribution of the alterations was unpredictable, often strongly focal, most often in continuation of the tumour. In one specimen, the margins of resection were involved. The extent was largest in bladders with poorly differentiated tumours. A temporal relationship between *in situ* carcinoma and invasive carcinoma could not be shown, as regards tumour size and duration of clinical symptoms. The morphology of the changes is discussed seen in the light of variations in the normal epithelium lining the lower urinary tract. Minor degrees of atypia of the epithelium showed rather bad reproduction. The dominating occurrence of carcinoma *in situ* in bladders with poorly differentiated tumours may either be a manifestation of a biological difference between tumours of different grades of differentiation or that the morphological criteria used correspond to the *in situ* type of poorly differentiated tumours.

Key words: Carcinoma *in situ*; urinary bladder; primary carcinomas.

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Carcinoma *in situ* of the lower urinary tract was first observed in bladders removed on the indication of primary carcinoma (14, 18). These changes were related to the well-known multifocality and tendency to a recurrence of tumours in this region. Some years later an investigation was commenced in which a group of industrial workers participated (16) who had been exposed to para amino diphenyl, a compound which shortly before had shown to be a potent carcinogen to the bladder. In several cases it was shown that the neoplasia passed through a clinically silent stage during which malignant cells were shed in urine and biopsy from the macro-

scopically normal bladder mucosa showed carcinoma *in situ*. According to a later report, a similar course was observed in patients without known carcinogenic exposure (23) The purpose of this investigation has been, on the basis of a consecutive material of cystectomy specimens, to examine the incidence, extent, and localization of carcinoma *in situ* and to look for a possible correlation between this and the histology of the tumours.

MATERIAL AND METHODS

The consecutive series comprised 46 patients who in the period 1 February 197? until 31 January 1974 underwent cystectomy in the Department of Urology Odense Hospital. One patient was excluded because of a simultaneously occurring adenocarcinoma of the prostate gland. Two patients were excluded due to imperfections in the histological technique. The remaining 43 patients were suffering or had been suffering from primary bladder cancer The series comprised 37 men and 6 women. The average age was 61 years with no sex difference. The males underwent cystoprostatectomy the females cystectomy Lymph node resection was not done. The duration of symptoms varied from 2 weeks up to 5 years. The median length was 6 months. Eight patients underwent resection of bladder tumour and 6 patients were irradiated before cystectomy Prior to cystectomy 1 patient had received cytotoxic therapy with Triethyleneglycol diglycidylether Epodyl® a compound used locally Three bladders contained no tumour at the time of cystectomy One of these was irradiated, one was resected, and one wa both irradiated and resected. The bladders were delivered to the Department of Pathology unopened and unfixed Immediately after arrival they were opened in the midline of the anterior wall and stretched out on a cork-plate The force by which the stretching-out was done could not be standardized After photography the bladders were fixed in 4 per cent aqueous formalin for at least 48 hours The cork-plate was divided into fields, each measuring 3 × 1 centimeter From each field, at least one tissue block was made and numbered corresponding to the co-ordinates of the field. By means of these the localization of the microscopical slide could be found on the photograph made of the fixed bladder the photograph being divided like the cork-plate by means of a screen, Fig. 1 Using this method of cutting, w obtained these from every one centimeter in the vertical direction of the system of co-ordinates and continuous representation in the horizontal direction. On an average 47 tissue blocks were prepared from each



Fig 1 Urinary bladder stretched out and fixed on a cork-plate. The photograph is made through a screen divided like the cork plate, the system of co-ordinates corresponding to the numbering of the specimens. Each field measures 3 × 1 cm.

bladder After embedding in paraffin wax and staining with haematoxylin-eosin, the microscopical slides were studied by two of us (NKJ and HS) Rather frequently it was necessary to make new slides from the blocks because the epithelium had fallen off during cutting in the microtome. The tendency to falling-off seemed more pronounced in dysplastic epithelium We were not successful in every case to have slides with continuous epithelial lining.

The 40 tumours were classified in accordance with *Bergkvist et al.* (3) In the present paper however we only have distinguished between highly differentiated tumours corresponding to grade 2 B and poorly differentiated tumours corresponding to grades 3 and 4 The grade 2 B was employed in cases of tumours with cellular deviation of more than moderate but not pronounced degree where there was no doubt about the loss of polarity though some remained.

Areas of the mucosal surface not occupied by tumour but at histological examination showing a definite polymorphia of enlarged nuclei with abnormal chromatin structure were considered carcinoma *in situ* Fig. 2. These alterations were often followed by reduced cohesion between cells. This, however was not used as a criterion. Dysplastic epithelium containing a connecti tissue stalk was regarded as tumour These criteria are in accordance with those set up by other authors (7 17 20)

The quantitative results are given as a percent based upon the proportion between the number of blocks with the alterations concerned and the total



Fig 2 Carcinoma *in situ* of the transitional epithelium of the bladder Haematoxylin-eosin, × 280

number of blocks in which such changes might be present. The figures are examined by means of the Mann-Whitney Rank Sum Test for unpaired data (22) Significance limits for the lesser rank sum for $p = 0.05$ originate from previously published tables (6)

RESULTS

Twenty-six out of the 43 bladders contained areas of the epithelial lining thus fulfilling our criteria of carcinoma *in situ* Among these were the three bladders which did not contain tumour at the time of surgery In addition, three patients in this group had previously had a bladder tumour resected but recurrence followed. The extent of carcinoma *in situ* ranged from 2 to 81 per cent with an average of 20 per cent. The localization of the alterations could be divided into five groups which reflect the variations in the distribution of the alterations. *Group I* contained the cases in which carcinoma *in situ* was found as a regular border encirculing the tumour Such a border was found in 10 out of 23 cases. In *group II* which comprised two cases, the border was in continuation of the tumour but irregular tongue-shaped. *Group III* comprised the cases which, besides a border contained isolated islands of carcinoma *in situ* Such islands were found in 8 cases. In *group II* which comprised three cases, nothing but isolated islands of *in situ* carcinoma were observed. Finally *group* 1 was composed of cases which did not contain tumour at the time when cystectomy was performed In one case, carcinoma *in situ* was found in an ureteral resection border

Among the 40 tumours investigated, one was predominantly an adenocarcinoma which could not be classified according to the criteria mentioned. The 39 predominantly

TABLE 1 *The Relative Extent of 39 Urinary Bladder Tumo s Divided Accord ng to Grade of Malignancy*

	Per cent of disposable mucosal rea					
	3	6.6	14.6	41.9		
11 highly differentiated tumours	4.5	9 1	14 7	76 5		
	5.5	11.5	28 6			
	1.9	5.9	9.6	17 1	23 4	44 1
	2.0	6.0	10.4	21.6	5 0	55 3
28 poorly differentiated tumours	2.3	6.8	10.9	23.1	25 7	77 1
	5.6	7 1	11 9	23.2	5	
	5.8	8 5	14.3	23.3	9	

Rank sum of the small group 212
Rank sum of the large group: 568.
Significance limits for lesser rank sum with $p = 0.05$ 157 283
The extent given in per cent of 39 urinary bladder tumours d ided in grades of malign ncy according to Bergk lit et al The per cent given was based on the number of fields, each measuring 3 l centimetre covered by tumour related to the total number of fields which was covered by the whol bladder The figures of each group were ranked and examined by the Mann-Whitney rank sum test for unpaired data.

TABLE 2. *The Relative Extent of Tumours in 40 Urinary Bladders 17 Without and 23 With Carcinoma* In Situ

	Per cent of disposable mucosal area					
17 tumours without carcinoma *in situ*	4.3	6.6	10.4	14 6	27.9	44 1
	5.3	7 1	11.5	21 6	28 6	76.5
	6 0	8.3	12.7	25.0	41.9	
23 tumours with carcinoma *in sit*	1.9	5.6	9 1	14.3	23.2	25 7
	2.0	5 8	9 6	14 7	23.3	55.3
	2.3	5.9	10.9	17 1	23 4	77 1
	3..	6 8	11.9	23 1	25 7	

Rank sum of the small group 377
Rank sum of the large group: 443
Significance limits for lesser rank sum with p = 0.05: 276–421
The extent given in per cent of 40 urinary bladder tumours in 17 bladders without and 23 bladders with carcinoma *in situ* outside the tumours. The per cent was based on the number of fields, each measuring 3 × 1 centimetre covered by tumour related to the total number of fields which was covered by the whole bladder The figures of each group were ranked and examined by the Mann-Whitney rank sum test for unpaired data.

urothelial tumours included 11 highly differentiated tumours. One of the latter invaded the muscle of the bladder wall, while this applied to 22 of the poorly differentiated tumours. The extent of the tumours varied from 2–77 per cent with an average of 19 per cent. Table 1 shows the extent of the tumours grouped according to their grade of differentiation. There is no significant difference ($p > 0.05$) between the extent of highly and poorly differentiated tumours. The extent of tumours in 23 bladders with carcinoma *in situ* is in Table 2 correlated to the extent in bladders without. The values do not differ significantly ($p > 0.05$) Table 3 shows the extent of carcinoma *in situ* related to the

TABLE 3 *The Relative Extent of Carcinoma* In Situ *in 39 Urinary Bladders With Tumour Divided According to Grade of Malignancy*

	Per cent of disposable mucosal area					
11 highly differentiated tumours	–	–	–	13.8		
		–	–	14.0		
	–		10.0			
28 poorly differentiated tumours	–	–	2.9	9.5	16.7	62.5
	–	–	4.2	9.5	19 3	68.7
	–	–	6.5	10.8	20 8	80.9
	–	1.8	7.3	12.5	23 8	
		1 8	8.2	15 9	33 4	

Rank sum of the small group 154
Rank sum of the large group 626.
Significance limits for lesser rank sum with p = 0 05 157 283
The extent of carcinoma *in situ* given in per cent in 39 urinary bladders with tumours divided in grades of malignancy according to Bergkvist et al The per cent was based on the number of fields, each measuring 3 × 1 centimetre covered by carcinoma *in situ* related to the number of fields without tumour from the bladder concerned. The figures of each group were ranked and examined by the Mann-Whitney rank sum test for unpaired data. 8 bladders in each group did not contain carcinoma *in situ*

TABLE 4 *The Relative Extent of Carcinoma In Situ in 40 Urinary Bladders With Tumours Related to the Duration of Urinary Tract Symptoms*

	Per cent of disposable mucosal area					
	–	–	–	8.2	14.0	33.4
Short duration of symptoms, 16 patients.	–	–	4.2	10.0	20.8	
	–	–	7.3	13.9	23.8	
	–	–	–	2.9	10.8	19.3
	–	–	–	6.3	[illegible]	62.3
Long duration of symptoms, 24 patients.	–	–	1.8	9.5	13.8	68.7
	–	–	1.8	9.5	16.7	80.9

Rank sum of the small group: 329
Rank sum of the large group 491
Significance limits for lesser rank sum with $p = 0.05$: 257–399
The extent of carcinoma *in situ* given in per cent in 40 urinary bladders with tumours. The patients were classified according to duration of symptoms from the urinary tract shorter or longer than 6 months which was the median value in the present investigation. The per cent was based on the number of fields, each measuring 3 × 1 centimetre covered by carcinoma *in situ* related to the number of fields without tumour from the bladder concerned. The figures in each group were ranked and examined by the Mann-Whitney rank sum test for unpaired data. Carcinoma *in situ* was not found in 7 patients in the first group and 10 patients in the second group.

degree of differentiation of 39 predominantly urothelial tumours. The extent of carcinoma *in situ* is significantly greater in bladders with poorly differentiated tumours. The extent in three bladders without tumours was 4.1, 24.4 and 27.9 per cent. The extent of *in situ* carcinoma in 16 patients with a history of lower urinary tract symptoms shorter than the median duration of symptoms was not significantly larger ($p > 0.05$) than that in the 24 patients with a longer history, Table 4. In this calculation, patients without tumour are excluded.

DISCUSSION

The morphological alterations on which we have based our diagnosis of *in situ* carcinoma correspond to those on which other authors have based diagnosis of atypia of the most severe degree, by some named severe epithelial atypia (4), by others intra-epithelial epithelioma (7), and flat intra-epithelial neoplasia (2). Most often, the term *in situ* carcinoma seems to be used. Minor degrees of epithelial alterations such as atypical hyperplasia (7, 11, 20) were not used in the present work as their diagnostic reproduction was not sufficiently good in our hands. The normal transitional epithelium shows marked morphological variation. The thickness depends on the dilatation of the bladder, varying from 3–8 cell-layers. There is no characteristic basal layer or differentiation. The epithelial changes in the lower urinary tract are estimated solely on the basis of polarity, polymorphia and structure of the nucleus. The problem about the gradual transitions between the different grades of atypia has been pointed out by other authors (4). Artificial alterations were more frequent and less constant than otherwise observed, for instance in squamous epithelium. Previously we mentioned the tendency of the transitional epithelium to desquamate. In this respect, the frequent presence of pyknotic changes should be added which made the evaluation of chromatin content difficult, a fact which is well-known from the cytologic literature where such cells are described as "decoy-cells" (24). In several patients in this series the epithelial lining of the ureters was built up of many layers of rather small cell. This hypercellular appearance may have been brought

about because stretching of the ureters was less than that of the bladder wall. Not infrequently we found that the nuclei of the urethral epithelium varied in size without showing abnormalities in the structure of chromatin. Such changes were often seen in the transitional zone between squamous and transitional epithelium. Alterations of the epithelial lining of ureters and urethra were very often subjects of discussion. Cases of doubt were not included.

Areas with *in situ* carcinoma were focal with rather abrupt transitions from normal epithelium. In spite of the wide distribution of the changes, a biopsy may not contain diagnostic areas. According to a publication based on multiple, randomly selected bladder biopsies from patients with carcinoma of the bladder (7) the occurrence of changes of the epithelium was rather frequent, varying from atypia to *in situ* carcinoma. The latter was most often found in bladders with infiltrating tumours but at a frequency lower than that observed in studies based upon cystectomy specimens (4) The distribution of these changes was completely unpredictable (12)

Several authors have directed the attention to the resection lines of the cystectomy-specimen. During investigation of 410 ureters, *in situ* carcinoma was found in 20 (21) while another author (19) found 9 such carcinomas in 60 ureters. Both authors reported the later appearance of tumour in the anastomosis. By means of fresh-frozen-section biopsies obtained at the time of partial or total cystectomy indicated by vesical carcinoma in 231 patients, 18 cases of *in situ* carcinoma and 20 cases of tumours in the ureters were found (5) In an autopsy series comprising 33 men who died suffering from cancer of the bladder 6 cases of *in situ* carcinoma of the urethra were found (8) In another series, comprising 45 cystectomy specimens 8 cases of carcinoma *in situ* of the membranous or prostatic urethra were found (4) In the present study we only found *in situ* carcinoma of the ureter in one case and no examples of urethral localization. The 11 bladders with well-differentiated tumours comprised three with *in situ* carcinoma while such changes were found in twenty out of the twenty-eight poorly differentiated tumours. The same relation between the grade of the tumour and the incidence of carcinoma *in situ* has been noticed by others (4) The quantitative extent was greater in bladders with poorly differentiated tumours. These findings either suggest a biological difference between tumours of varying grades (7) or they open up the possibility that different types of *in situ* carcinoma may exist. The prognostic evaluation of carcinoma *in situ* is complicated by the simultaneous occurrence of poorly differentiated and therefore more aggressive tumours.

The significance of *in situ* carcinoma of the bladder is not definitely clarified. In the investigations mentioned in which persons exposed to carcinogen participated, the probability was convincingly advocated that carcinoma *in situ* precedes the infiltrating carcinoma. A group of 13 industrial workers participating in the latter trial in whom the diagnosis carcinoma *in situ* had been established in the period 1954 until 1967 were seen at follow-up. By 1968 (10) 7 of these had developed invasive carcinoma while the abnormal cytologic findings persisted in 4 The latent period lasted for a few months up to eight years. Several investigations are concerned with *in situ* carcinoma in bladders without pre-existing or existing tumours and without known carcinogenic exposure. In a retrospective study of 62 such cases (23) 37 of the patients developed invasively growing cancer The same course of events is described prospectively in several publications (1 9 13 15 17 25) The present series does *not* comprise any cases known to have been exposed to carcinogenics. Six patients had previously been treated by irradiation. In 4 of these *in situ* carcinoma was found. *One patient* had been treated with a cytotoxic compound and, at the time of operation, *in situ* as well as invasive carcinoma were present. The morphological features of the epithelial changes in the five patients did not differ from those in the remaining patients in the

series. Prior to operation, three bladders without tumours had shed tumour cells. The possible chronological relationship between *in situ* carcinoma and invasively growing tumour could not be established. Bladders with *in situ* carcinoma did not contain tumours smaller than those in bladders without. The extent of *in situ* carcinoma was not larger in bladders giving symptoms only for a short time. Carcinoma *in situ per se* however gives only few and vague symptoms (2)

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TYPES OF METAPLASIA IN FORTY UROTHELIAL BLADDER CARCINOMAS

A Systematic Histological Investigation

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Starklint, H., Kjærgaard, J & Jensen, N K. Types of metaplasia in forty urothelial bladder Carcinomas. A systematic histological investigation. Acta path. microbiol. scand. Sect. A, 84 137–142, 1976

In a systematic study of bladders from consecutively cystectomized patients, 40 primary urinary bladder carcinomas were examined with regard to the type of differentiation. Metaplastic areas were found in 24 of the tumours. A positive reaction of keratin to Kreybergs stain was required for the definition squamous metaplasia. Squamous areas were found in 16 of the tumours. In 4 tumours there was, in addition to squamous metaplasia, glandular metaplasia which was defined as definite glandular tissue with dysplasia of the epithelium. In 4 tumours, glandular metaplasia was also present in addition to the urothelial carcinoma. In 18 cases, metaplastic changes were present in the luminal portion of the tumour from which the biopsy material and desquamated material originated. The occurrence of metaplasia was related to the degree of differentiation of the urothelial part of the tumour Metaplastic changes were most frequent in the poorly differentiated tumours. The biopsies obtained pre-operatively permitted of the diagnosis metaplasia being made in 15 cases, while the cytological material suggested metaplastic changes in 4 cases only The biological function of metaplasia is unknown. The possibility that these areas will react differently to radiation and chemotherapy is present and the frequency of such changes would suggest that their importance should be studied by their registration as mixed forms as stated by WHO.

Key words Bladder carcinoma differentiation.

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It is well-known that different types of differentiation occur in urothelial tumours and the biological function of metaplasia is unknown. The WHO classification from 1973 (11) advises the classification of mixed forms as variants of transitional cell carcinoma. Squamous areas occur frequently but regions of glandular tissue are also well-known. Tumours can only be classified as either squamous cell carcinoma or adenocarcinoma where these cell types occur as the only phenomenon. These tumours are rare, particularly adenocarcinoma.

We have studied the occurrence of metaplastic areas and related these to the degree of malignancy of the tumour This was done by means of a material, collected with particular reference to a close and optimal light microscopic examination, requiring the prep-

TABLE 1 *Age and Sex Distribution of 40 Patients Subjected to Cystectomy for Primary Urinary Bladder Cancer*

	No. of patients with bladder cancer distributed according to age				Total
	< 49	50–59	60–69	70–79	
Women	0	3	2	1	6
Men	4	10	16	4	34
Total	4	13	18	5	40

aration of a large number of sections from each bladder and removed according to a predetermined plan.

MATERIAL AND METHODS

The material consists of 40 tumour containing bladders removed at cystectomy by the Urological Department of the Odense University Hospital in the period 1 2. 1972–31 1 1974 Six of the patients were women. The age distribution of the series can be seen from Table 1 The average age was similar in the two sexes, 61 years. The bladders were brought to the Institute of Pathology immediately after removal, where they were stretched out, photographed and fixed in 4 per cent aqueous formalin, as described in an earlier paper (15) The tumours were classified macroscopically as papillary sessile or ulcerative. The latter histologically contained both of the former The macroscopic evaluation was based entirely on observation of the operative specimen and was carried out without knowledge of the endoscopic picture. The system of section removal consisted of 1 section being taken from each field measuring 3 × 1 cm. More than one section was often removed from fields containing tumours and several were always removed from fields where the thickness of the wall in the area of the tumour was greater than the width of the capsule used. After embedding in paraffin and cutting the slides were stained using haematoxylin-eosin and then examined. The tumours were graded using the criteria suggested by *Bergkvist et al.* (2) In this study the grading 2 B was employed for tumours with cellular deviation which was more than moderate but not pronounced and where there was no doubt as to the loss of polarity but where some remained. The staging of the tumours was carried out after the classification by U.I.C.C. This combination of grading and staging is recommended by the Danish Urological Society (17) When the slides stained with haematoxylin-eosin showed differentiation other than urothelial supplementary staining was carried out using the method suggested by *Kreyberg* for the staining of keratin and mucopolysaccarides (10) Staining was carried out at pH 2.7 The keratin is stained a bright red, while the mucosubstances are coloured bluish-green. If the areas could not be characterized by this stain further staining was carried out with PAS-stain for neutral mucosubstances and Lendrum stain for fibrin. Attention was given in particular to the luminal cell population of the tumour the areas from which the biopsies and cytological material originated. Preoperative biopsies and cytological material in the form of fluid from bladder lavage and/or urine were available from all the patients. These were examined for the occurrence of the type of differentiation observed in the operative specimen. The biopsies were stained in the same manner as the operative specimens. The examination was performed with knowledge of the differentiation of the operative specimen

Squamous metaplasia was classified from one or both characteristics: the presence of intercellular bridges or the occurrence of bright red material in or on the surface of the cells following staining according to the method of Kreyberg Glandular metaplasia was defined as the presence of undoubtedly glandular tissue with dysplasia of the epithelium with or without mucous production. In those cases where none of the above mentioned types of differentiation were present in the malignant tumour it was classified as dedifferentiated urothelium.

RESULTS

The areas covered by the 11 highly differentiated tumours corresponding to grade 2 B and the 28 poorly differentiated tumours corresponding to grades 3 and 4 can be seen from Table 2 The extent of the tumour as a measure of the size varied from 2 77 per cent of the available mucosal area. The percentage was based on the number of fields which were

TABLE 2. *The Extent of the 39 Bladder Tumours in Percentage of the Available Mucous Membrane in the Respective Urinary Bladders Grouped According to the Degree of Malignancy*

	Extent of tumour in per cent								Total
	< 10	11 20	21–30	31–40	41–50	51–60	61 70	71–80	
Tumour grade II B	5	3	1	0	1	0	0	1	11
Tumour grade III & IV	12	4	9	0	1	1	0	1	28
Total	17	7	10	0	2	1	0	2	39

TABLE 3 *The Macroscopic Appearance of 39 Urinary Bladder Carcinomas in Relation to the Stage and Grade*

Tumour		Macroscopic appearance of tumour			No.
Stage	Grade	Papillar	Sessile	Ulcerative	
IS & P1	II B	7	2	1	10
	III & IV	6	0	0	6
P2 & P3	II B	0	0	1	1
	III & IV	12	5	5	22
Total		25	7	7	39

TABLE 4. *Type of Differentiation of 40 Primary Urinary Bladder Carcinomas the 39 Graded According to* Bergkvist et al.

	Type of differentiation of bladder carcinoma			
	Urothelial	Urothelial Squamous	Urothelial Squamous Glandular	Urothelial Glandular
Grade II B	9	2	0	0
Grade III & IV	7	14	4	3
Total	16	16	4	4 *

One tumour not graded.

the site of tumour formation in relation to the total number of fields each of a size 3 × 1 cm obtained from the bladder One of the tumours in the material was almost entirely adenocarcinoma, and this could not be graded using the criteria of Bergkvist and co-workers.

Thirteen of the tumours classified macroscopically as papillary were found to show superficial growth on microscopy (Table 3) These could be divided into 7 highly differentiated and 6 poorly differentiated tumours, while the 12 papillary tumours that

TABLE 5 *Comparison between Demonstrable Type of Differentiation of 40 Primary Urinary Bladder Carcinomas and the Preoperative Histological and Cytological Examination*

	Type of differentiation of bladder carcinoma			
	Urothelial	Urothelial Squamous	Urothelial Squamous Glandular	Urothelial Glandular
Operative specimen	16	16	4	4
Luminal surface	22	14	0	4
Biopsy	25	12	0	3
Cytological material	36	4	0	0

invaded the muscle layer were all poorly differentiated. Two of the group of sessile tumours were highly differentiated. Among the ulcerative tumours there was only one highly differentiated with superficial growth. The others invaded the muscle layer

Table 4 shows the occurrence of the three various types of tumour tissue present in the 40 tumours, classified according to the grade of differentiation of the urothelial part. This could only be determined with regard to 39 tumours as one contained too small urothelial areas. The occurrence of metaplasia was most frequent in the poorly differentiated and deeply invasive tumours. Areas with squamous metaplasia were found in 2 highly differentiated tumours, while all cases of glandular metaplasia occurred in poorly differentiated tumours. The metaplastic areas were not evaluated quantitatively. However the site of these changes was studied with particular reference to the luminal surface of the tumour areas from which the biopsies and exfoliated material originated. Metaplastic areas were found in 24 tumours with the methods used, while in 16 tumours it was only possible to demonstrate urothelial carcinoma. It can be seen from Table 5 that the pre-operative biopsies from 25 patients showed no signs of metaplastic areas. Squamous metaplasia could be demonstrated in 12 biopsies. Only 8 of the corresponding 12 tumours could be registered as squamous on the luminal surface. The 4 remaining biopsies originated from tumours with squamous metaplasia in the deeper lying parts of the tumour The other 6 tumours with luminal squamous metaplasia did not have such changes in the biopsies. Two tumours had squamous metaplasia which could not be seen either on the surface or in the biopsies

Squamous metaplasia was suspected following cytological examination in 4 cases. The corresponding 4 tumours were registered as squamous metaplasia on the luminal surface. The group of tumours showing all three various types of tumour tissue did not permit of a pre-operative diagnosis of metaplasia being made Three of the 4 cases with glandular metaplasia contained such changes in the biopsy and luminal surface of the tumour The cytological material did not reflect the histological changes found.

DISCUSSION

The present series is selected and consists of cases where the tumour was confined to the bladder or surrounding soft tissues and thus operable Similarly the condition of the patients was such that prolonged surgery was possible The age distribution and stage of tumours are representative of such patients.

More than half of the tumours were papillary They represented both highly and poorly differentiated forms (Table 3) It is remarkable that the group of papillary tumours contained such a large number of poorly differentiated and deeply infiltrating tumours. Two of

the sessile tumours were highly differentiated. The macroscopical evaluation of the tumour as a guide to the degree of malignancy was therefore of little value.

The 40 tumours were all considered primarily as being urothelial with larger or smaller areas of the other two types of differentiation (14). The limitation of the areas of squamous metaplasia was difficult. The occurrence of epithelial pearls and intercellular bridges was rare. This is an expression of the fact that these morphological characteristics correspond to highly differentiated planocellular tumours. The use of staining for keratin was often of help but can be a source of some error inasmuch as several tissue elements are stained red, even though to a varying degree. Thus necrotic tissue is stained red, which in connection with the presence of nuclear residue could give the false impression of keratinization. The study showed a discrepancy between the evaluation of the surface of the biopsy and the luminal portion of the tumour in the corresponding operative specimen. This discrepancy reflects an uncertainty in the diagnosis of planocellular metaplasia. The glandular metaplasia was a limited unit, characterized by definite glandules, which were dysmorphological with or without mucous production. Cases of epithelial atypia in cystic cystitis outside of the tumour were not included. Cystic cystitis was found in all of the operative specimens to varying extents.

Metaplasia of the epithelial lining of the bladder is known from a large number of non-neoplastic conditions. Chronic irritation can give rise to both squamous and glandular metaplasia (12). The ability of the epithelium of the bladder to form keratin under special circumstances can be presumed to be caused by a morphological and functional similarity to stratified squamous epithelium at other sites in the organism. It has been shown that the layer which forms an impermeable membrane between the urine and epithelium has properties that histochemically and ultrastructurally correspond to those of keratin (7). The origin of glandular epithelium is now presumed to be epithelial inclusions in the lamina propria, such as cystitis cystica cystitis cystica glandularis and cystitis glandularis (16, 18). Such changes are in the early stages, extremely frequent (1). Opinions differ considerably as to the premalignant potential of both planocellular and glandular metaplasia (15). The occurrence of planocellular and glandular areas in a neoplasm would suggest that the changes are of a metaplastic nature (6). If the origin had a direct relationship to the embryology of the bladder then mixed forms would not be expected, such as is the case here. Metaplastic areas in the urothelial tumours were mainly found in this material in the poorly differentiated tumours, such as was the case in the larger materials (3). It has been stated (6) that tumours with metaplastic areas do not differ biologically to corresponding differentiated urothelial tumours without such areas. This conclusion could be better verified providing changes were registered as recommended by WHO. A grading of the degree of dysplasia of the non-urothelial elements as suggested by Bergkvist and coworkers (2) according to the same scheme as grading of urothelial dysplasia is done would be informative, if it was carried out, but would not give information as to the behaviour of metaplastic areas. While the planocellular areas appear to partly follow the degree of malignancy of the urothelial tumour this has not been demonstrated with regard to adenocarcinoma. Studies of adenocarcinoma have demonstrated a poorer prognosis for patients with this type of tumour than for those with urothelial carcinoma (8) and that radiation therapy is of very little value (16). Therefore, it is possible that areas of adenocarcinoma will respond differently to non-surgical tumour treatment than other parts of the tumour.

The possibility of a diagnosis of the described changes being obtained by biopsy are not so good. However in 12 of the 20 cases of squamous metaplasia in the tumour corresponding areas were found in the biopsy while 3 of 8 cases with glandular metaplasia

could be diagnosed from the biopsy The 14 cases of planocellular differentiation on the surface was only reflected in the cytological material from 4 patients. This is in agreement with the good correlation between cytological and biopsy evaluation of the degree of differentiation of urothelial tumours (4 9). An investigation based on 411 biopsies from the bladder (5) showed a far lower frequency of metaplastic changes than that demonstrated here, a difference which, to a major degree, can be explained by the method of examination.

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KURLOFF CELLS

1 Histochemical Characteristics of the Kurloff Bodies

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Kortelainen, L. & Korhonen, L. K. Kurloff cells. 1 Histochemical characteristics of the Kurloff bodies. Acta path. microbiol. scand. Sect. A, 84 143–153 1976.

Kurloff cells, characterised by a large inclusion body in ill-defined lymphoreticular cells, were induced in male guinea pigs by oestrogen administration. The inclusion body material was characterized by way of light and electron microscopy and histochemical staining reactions. Nucleo-protein were not detected, but certain tests for amino acids were observed and, at the peripheral parts of the inclusion bodies, phospholipid tests were positive. However forced proteolysis of lipid extractions failed to abolish the reactions for detection of carbohydrate-rich compounds in the inclusion bodies thus confirming that their main characteristics were those of glycoproteins. Series of histochemical tests demonstrated the presence of periodate-reactive 1,2-glycol groups and sulphate radicals. Periodate-reactive material and sulphomucins represented two separate substances, as demonstrated by extractions and combined staining tests. Some characteristics common with chondroitin sulphates were observed but, on the other hand, the observed differences suggested different type of sulphation. The inclusion body failed to show any hydrolytic, dehydrogenase or oxidative enzyme activities, thus being metabolically inactive, although the cells showed certain enzyme activities in their cytoplasm and cell organelles.

Key words Kurloff cell oestrogen effect lymphoreticular system histochemis electron microscopy

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Kurloff bodies are large intracellular inclusions which appear in ill-defined lymphoreticular cells to pregnant guinea pigs or in males receiving oestrogens. Cells containing these inclusion bodies are found in the peripheral blood, bone marrow spleen and thymus. The nature and significance of the Kurloff cells and their inclusion bodies have been a source of considerable debate since their discovery by *Kurloff* (1889) and *Foa & Carbone* (1889). Physiologically these cells are found in female guinea pigs as well as in foetuses and newborn animals. Their number is known to increase during pregnancy and they may also be induced in adult males by oestrogen administration. The inductive action of oestrogens was suggested by *Alexeieff & Joukoff* (1928) and later it was clearly demonstrated by *Ledingham* (1940).

Charles & Nicol (1961) also observed hyperimmunoglobulinaemia in guinea pigs producing Kurloff cells and the investigations by *Christensen et al* (1970) and *Revlov et al.* (1970) also suggested the participation of these cells in the immunological reaction.

Marshall et al. (1971) demonstrated the cytotoxic effect of the Kurloff body material on macrophages and the presence of immunologically identical material in the placental trophoblasts and the foetal endothelium of the placental labyrinth. This investigation, as well as that of *Warren et al.* (1971) suggests that Kurloff cells may play a part in preventing immunological damage to the target cells via the defence systems of the host organism.

The process resulting in the formation of the Kurloff bodies has been suggested to be one of intracellular secretion, intracellular parasitism, phagocytosis of other cellular material erythrophagocytosis, nuclear degeneration etc. theories which have been reviewed by *Smith* (1947) *Pearse* (1949) and *Marshall & Swettenham* (1959) have studied the histochemistry of the Kurloff bodies more closely obtaining partially controversial results. *Muir & Marshall* (1961) reported some preliminary biochemical characteristics of the Kurloff body material *Dean & Muir* (1970) published an extensive analysis of the spleen extract obtained from oestrogen-induced guinea pigs.

Since the Kurloff bodies have not been isolated in a pure state biochemical analyses of these require extracts of whole spleen or other suitable organs. Therefore, it appeared useful to study the Kurloff body material in greater detail by way of histochemical staining reactions in order to characterize only the inclusion body material without the interference of other components of the organ under observation. The present study concerns the histochemical and morphological characteristics of the Kurloff cells and their inclusion bodies In the first part the inclusion body material was characterized by means of various histochemical staining reactions, in an attempt to make the present incoherent information more complete and to revalue some of the controversial findings presented in the literature. In the second part (*Kortelainen & Korhonen* ibid) additional characteristics of the cells have been investigated in order to elucidate the origin of these cells.

MATERIAL AND METHODS

20 male guinea pigs, aged from 2 to 4 months, were used as test animals. They received three doses of 0.5 mg Oestradiol benzoate/100 g over a period of two weeks. Two to three weeks after the last injection, the animals were killed by N_2 suffocation and decapitation. Histological samples were collected from the spleen, thymus, liver lymph nodes and, occasionally from other organs. Spleen imprints were also used.

Tissue samples 10 per cent Neutral formaline was used as a routine fixative. Kurloff body material is ideally preserved in alcoholic fixatives methanol, Carnoy or 10 per cent formaline in 70 per cent ethanol were used for this purpose. Most of the histological and histochemical tests were performed on routine paraffin sections. Some histochemical reactions required the use of fresh unfixed samples for which purpose imprint preparations were used Electron microscope specimens were fixed in cacodylate-buffered glutaraldehyde and subsequently embedded in Epon-Araldite

A Reichert Ultramicrotome with diamond knives, was used to the preparations of the sections which were stained with uranylacetate and lead citrate and studied under a JEM 100 B Electron microscope.

Staining Methods and Histochemical Reactions

Routine staining procedures, for instance H E, Giemsa etc., were performed according to Manual of Histologic Staining methods (*Luna* 1968) The histochemical procedures were performed according to the instructions given by *Pearse* (1968) if not otherwise stated. Tests for carbohydrate-containing substances were performed according to *Spicer et al.* (1967) and *Korhonen & Mäkelä* (1971) The "pH signature" was checked according to *Lillie* (1948)

RESULTS

Kurloff cells were observed in male guinea pigs after a single intramuscular injection of oestrogen although injections often were repeated in order to obtain larger numbers of these cells. They were first observed in the spleen thymus, bone marrow and peripheral blood. Later single cells, or small groups of cells, were also seen in the sinuses of the liver and occasionally in the lymph nodes as well as in the capillaries of other organs. The light microscopic appearance corresponded to that previously reported the first detailed description being given by *Ledingham* (1940) The typical Kurloff cell is ovoid in form with



Fig. 1. An imprint-preparation of guinea pig spleen after an oestrogen induction of two weeks. The typical shape of Kurloff cells is seen. A crescent shaped nucleus is flattened at the margin of the rounded inclusion body. Some clear vacuoles are seen in the cytoplasm. Note a cell with two inclusion bodies (arrow). × 1000.

Fig. 2. A preparation similar to that in Fig. 1 but air-dried previous to fixation and staining. The Kurloff cells display shrunken inclusion bodies (arrows). × 1000.

Fig. 3. Sudan Black B in 70 per cent ethanol staining from a formalin fixed section of oestrogen-treated guinea pig spleen. Small Sudan Black-positive granules are seen surrounding the unstained Kurloff inclusion bodies. × 1000.

a diameter ranging from 10 to 14 µm in imprint preparations. Such cells possess one large spherical inclusion body by which the sickle-shaped nucleus is pushed towards the periphery of the cell (Figs. 1–2).

The margin of the inclusion body often displays small vacuoles. In the spleen and bone marrow cells with smaller inclusion bodies more than one per cell, might occasionally also be seen. In preparations stained with H E of by other routine methods, the Kurloff cells were rather difficult to observe because the inclusion body approximates the size of an erythrocyte and often stains in a similar fashion. However, they are easily identified by means of e.g. Giemsa stain or the PAS reaction.

The morphology and the histochemical staining characteristics of the inclusion bodies varied according to the treatment of the sample. The inclusion bodies were destroyed and in fresh samples their content readily released by freezing, mechanical compression, or in hypotonic solutions. On the other hand, in formaldehyde or alcohol fixed preparations they were exceptionally stable.

Certain staining reactions were also dependent on the type of the fixative. PAS and PA-pD reactions were positive irrespective of the fixation method used, but tests for acid radicals, like AB or HID were only positive or greatly intensified if alcohol containing fixatives were used. In the electron micrographs, the electron density of the inclusion

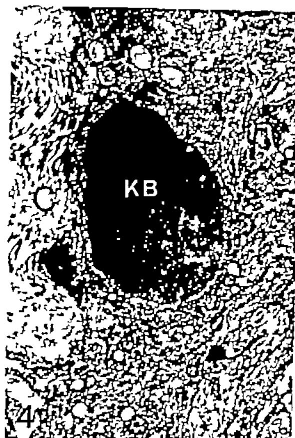




Fig 4 An electron microscope section of the guinea pig spleen. Fixation with phosphate buffered glutaraldehyde. A cell with a large inclusion body (KB) is closely associated with collagen fibres (C) × 10 000

Fig 5 A detail from the margin of the inclusion body fixed in cacodylate buffered glutar(di)aldehyde. Note the pale inclusion material (I) channels extending from it to the cytoplasm (arrows) and myelin figures (M) Some mitochondria (mi) and a nucleus ( ) are seen. × 40 000

body was greater after the use of phosphate buffered fixatives than after cacodylate buffered fixatives, no matter the thickness of the section (Figs 4–6).

The histochemical staining reactions for the detection of DNA, amino acids and lipids (Fig. 3) and the interpretation of these are given in *Table 1* where other histochemical results also are recorded.

Histochemical tests for carbohydrate-rich compounds (Table 1 IV) were greatly effected by the fixation method used. It applies to the majority of fixation methods that those based on the periodic acid oxidation of vicinal glycol groups were *positive but those demonstrating* acid radicals displayed positive reactions only if alcoholic fixatives such as absolute methanol Carnoy or 10 per cent formalin in 70 per cent ethanol had been used. The material in the inclusion bodies was periodate reactive giving a strongly positive PAS reaction and weakly positive PA-pD reactions. Negative results to be obtained if the sections in the Schiff reagent were treated without previous oxidation ("direct Schiff") excluded the participation of free aldehyde groups and oxidizing agents, etc in the PAS-reaction, while the acetylation-de-acetylation sequence confirmed the 1,2-glycol nature of the reactive groups.

Although lipids with double bonds obviously were present in the inclusion bodies,

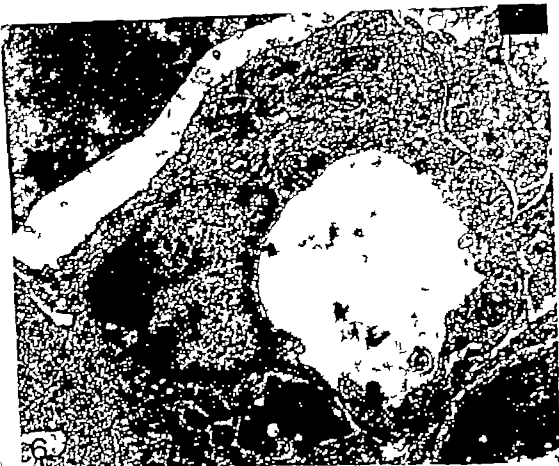


Fig. 6 If cacodylate buffered glutaraldehyde were used instead of the phosphate buffered glutaraldehyde, the inclusion bodies of Kurloff cell would be much less electron dense. Normal cell organelles, rough-surfaced endoplasmic reticulum, mitochondria, microfilaments and various vacuoles are seen. Note also myelin figures at the margin of the inclusion body and membrane-bounded channels continuing into cytoplasm (arrows) × 20 000

their positive interaction with the reaction as well as the retention of the PAS reaction after forced lipid extractions could be excluded on the basis of the tests mentioned (Table 1 1\ 3) These tests also included extraction in hot chloroform-methanol which remove all lipids, and in hot xylene acetic acid which is known to extract myelin-like materials which during the fixation process have been rendered partially insoluble (*Lillie* 1948)

Acid radicals in the inclusion bodies were not adequately demonstrated except after alcoholic fixations. This may explain the somewhat controversial observations presented in the literature In our hands, reactions by which to acid radicals were negative in formalin-fixed material but several reactions were positive if samples were fixed in alcoholic solutions. The selective extraction of material containing acid radicals obviously occurred in watery media, while the "neutral" carbohydrate-rich compounds displaying periodate reactivity were preserved.

Alcianophilia, HID reaction and Toluidine Blue metachromasia varied somewhat from cell to cell These phenomena were generally more intense in the peripheral parts of the inclusion bodies, suggesting the heterogeneous nature of the inclusion body material. The mixed diamine reaction at pH 3.8 gave nega

TABLE 1 *The Main Histochemical Characteristics of the Inclusion Bodies in Kurloff Cells*

Histochemical tests	Results	Notes on the interpretation
I *Nucleic acids*		
Feulgen reaction (according to *Pearse* 1968)	Negative in the inclusion bodies, positive in the nucleus.	DNA not present in the inclusion bodies.
Basic nuclear stains (except Toluidine Blue)	Negative in the inclusion bodies, positive in the nucleus.	
Pyronine (RNA-ase labile) (Brachet, according to *Pearse* 1968, and *Scott* 1967)	Negative in the inclusion bodies, weakly positive in the cytoplasm.	RNA present in the cytoplasm, but not in the inclusion bodies.
II *General tests for proteins and amino acids general staining characteristics*		
Millon (according to *Pearse* 1968)	Weakly positive in the inclusion bodies.	Tyrosine-containing proteins present.
Sakaguchi.	Weakly positive in the inclusion bodies.	Arginine-containing proteins present.
Azurophilia (e.g. Giemsa)	Positive in the inclusion bodies.	
Eosinophilia.	Unstained or very weakly stained in haematoxylin-eosin.	
Basophilia.	Unstained by most basic stains except Toluidine Blue	
III *Lipids and extraction tests*		
Sudan Black B in 70% ethanol.	Negative in the inclusion bodies, but positive droplets around it.	Neutral fats and strongly reactive phospholipids not present.
Oil Red O in ethanol-ether	Negative.	
Sudan Black B for masked lipids.	Negative.	
Baker's acid hematein. Elftman's controlled chromatin. Acid hematein after 24 hrs extraction in boiling methanol-chloroform.	Greyish-blue reaction in the inclusion bodies. Reaction not completely removed	Positive reaction indicates the presence of phospholipids, but as the material was not completely removed by extraction and as muco-substances are present, the reaction can be unspecific.
Performic acid Schiff (PFAS)	Positive in the inclusion bodies.	Suggest the presence of lipids with unsaturated bonds (phospholipid)
Plasmal reaction.	Weakly positive.	Acetal lipid possibly present.
Lugol.	Negative.	
IV *Carbohydrate-rich compounds* The staining characteristics of carbohydrate rich compounds in the inclusion bodies are easily modified by the fixation method used tests for the acid radicals requiring the use of alcohol-containing fixatives.		
a) Periodic acid, diastase-labile material PAS reaction after diastase digestion.	No effect.	Glycogen not present.
b) Characteristics of the periodate-reactive compounds Direct Schiff (without oxidation)	Negative	No reactive groups present without previous oxidation.
PAS	Positive in the inclusion bodies.	1,2-glycol groups present.
Acetylation-PAS	Negative	Reactive groups can be blocked by acetylation and unblocked
Acetylation-deacetylation-PAS	Positive, like PAS alone	by subsequent de-acetylation, confirming the 1,2-glycol nature of the reactive groups.

TABLE 1. (continued)

Histochemical tests	Results	Notes on the interpretation
Periodic acid-p-diamine (PA-pD)	Positive in the inclusion bodies.	Dialdehyde groups present after periodic acid oxidation, indicating the presence of galactose or fucose.
c) Characteristics of acid mucosubstances		
1. Sulphate radicals		
Alcian Blue at pH 1.0 (AB 1.0)	Positive in the inclusion bodies.	AB 1.0 CEC extinction at 0.5 M $MgCl_2$ concentration and HID reactions suggest the presence of sulphate radicals in the inclusion body material.
Critical Electrolyte Concentration (CEC)	Alcianophilia extincts at 0.5 M $MgCl_2$ concentration.	
High Iron Diamine (HID)	Positive in the inclusion bodies.	
Aldehyde Fuchsin (AF)	Negative.	
Ruthenium Red (RR)	Negative.	
Low Iron Diamine (LID)	Positive.	
Periodic acid-Low Iron Diamine.	Unchanged.	
Periodic acid-High Iron Diamine (PA-HID)	unchanged.	
Mixed Diamine	Negative.	
Meta-diamine blocking.	Blocks the alcianophilia.	
Toluidine Blue metachromasia.	Positive in the inclusion bodies.	
Colloidal Iron (CI)	Positive in the inclusion bodies.	
2. Carboxyl radicals		
Alcian Blue pH 2.5 (AB 2.5)	Positive in the inclusion bodies.	AB 2.5 reaction may indicate the presence of carboxyl radicals, but because of the presence of sulphate radicals, these cannot be separately identified. Single tests indicating the carboxyl radicals alone were negative.
HID-AB 2.5	Only HID reaction positive.	
Weak methylation-AB 2.5	Unchanged.	
Strong methylation-demethylation-AB 2.5.	Negative.	
3. Other characteristics		
AB 1.0 and AB 2.5 after testicular hyaluronidase or vibrio cholera sialidase digestion.	Unchanged.	Main characteristics of the material are not those of chondroitin-4- or chondroitin-6-sulphates or non-acetylated sialic acid.
PAS reaction in the fixed preparate after trypsin digestion.	Reaction in the inclusion body unchanged	Main characteristics of the inclusion material is not those of proteins.
PAS reaction after a series of lipid extractions according to Adams	Unchanged	Carbohydrate material is not closely bounded with the lipids.
PAS reaction after extraction in hot xylene acetic acid 3:7 (*Lillie* 1948)	Unchanged.	

tive results although control sections of intestine showed the well-known reaction pattern. Previous periodic acid oxidation failed also to show any reactive groups in Kurloff cells in the mixed diamine procedure. If ferric chloride were added in order to accelerate the oxidation in the staining solution the LID and HID methods would both give positive results in the inclusion bodies. These reactions remained unaltered by previous

TABLE 2 *pH Signatures*

Azure A	pH 1.0	2.0	3.0	4.0	5.0	6.0	7.0			
Kurloff bodies	0	0/+	0/+	0/+	+	++	+++			(violet from pH 2.0 if methanol fixed) pale yellow if formalin-fixed to pH 5.0 from this pH blue.
Erythrocytes	0	0	0	0	0	+	++			(yellow at pH 5.0 green from pH 6.0)
Nuclei, generally	+/0	+	++	++	+++	+++	+++			
Cytoplasm (liver)	0	0	0/+	++	+++	+++	+++			
Goblet cell mucin (intestine)	0	+	++	++	+++	+++	+++			
Orange G	pH 4.0	5.0	6.0	7.0	8.0	9.0	9.5	10.0	11.0	
Kurloff bodies	++	+/0	0	0	0	0				
Erythrocytes	++++	+++	++	+	+	+/0				
Nuclei, generally	0	0	0	0	0	0				
Cytoplasm (liver cell)	+	+/0	0	0	0	0				
Goblet cell mucin (intestine)	0	0	0	0	0	0				
Biebrich Scarlet										
Kurloff bodies			++++	++++	+++++	+++	0	0		
Erythrocytes			++++	++	++	0	0	0		
Nuclei, generally			+	0	0	0	0	0		(nucleolus stained up to pH 8.0)
Cytoplasm (liver)			+	0	0	0	0	0		
Fast Green FCF	pH 4.0	5.0	6.0	7.0	8.0	9.0	9.5	10.0	11.0	
Kurloff bodies	+++	+++	+++	+++	+++	+++	++	++	+	blue green
Erythrocytes		+	+++	++	++	+++	++	++	+	yellow green
Nuclei, generally			0	0	0	0	0	0	0	
Cytoplasm (liver)			+	+	+/0	0	0	0	0	
Goblet cell mucin (intestine)	+/0	0/+	0	0	0	0	0	0	0	

periodic acid oxidation, which may suggest that the periodate reactive groups were not closely related to the acid radicals (*Spicer* 1965).

The "pH signature" of the inclusion bodies was also tested (Table 2). It was found to be greatly affected by the fixation method used and so depend on the buffer and electrolyte concentrations. The difference between the erythrocytes and the inclusion body material was obvious.

Various histochemical methods by which to demonstrate dehydrogenases, cytochrome oxidase and hydrolytic enzyme activities were also tested. All tests were negative in the inclusion bodies, but some were positive in the cytoplasm of the Kurloff cells. A detailed description of these results is given in the second part of this study (*Kortelainen & Korhonen* ibid).

DISCUSSION

The negative tests for nucleoproteins excluded that the Kurloff inclusion bodies might be derived from degenerated material from the extruded nuclear lobe or other nuclear remnants, as suggested by *Leinati* (ref. by *Ledingham* 1940). RNA-labile pyroninophilia in the cytoplasm, also observed by *Pearse* (1949) and by *Marshall & Swettenham* (1959), suggested the presence of RNA which was confirmed by electron microscopy by which free ribosomes as well as rough surface endoplasmic reticulum could be seen in the cytoplasm of Kurloff cells.

Some staining reactions by which amino acids might be detected were weakly positive, as found also in previous studies (*Pearse* 1949, *Marshall & Swettenham* 1959). However, the inclusion bodies presented an exceptional resistance to trypsin digestion, in that they retained some PAS reactivity although other cellular material was digested or destroyed into a formless mass. Although the inclusion bodies contained some protein material, the main characteristics of these were not like those of proteins.

Small granules stained with Sudan Black B, thus suggesting the presence of lipid material, as well as the positive performic acid Schiff and plasmal reactions. Although the presence of mucosubstances in the chromation tests for phospholipids was questionable, histochemical tests at a light microscopic level considered together with the electron microscopic observation of numerous myelin figures in the peripheral parts of the inclusion bodies, suggested the presence of some lipid material, possibly of phospholipid nature. Once again, forced lipid extraction methods failed to destroy the PAS reactivity of the inclusion bodies and, accordingly, the main characteristic of the inclusion material was not of a lipid nature.

The variable behaviour of neutral mucosubstances and acid sulphomucins in the fixatives indicates that at least these two different types of mucosubstances were present in the inclusion bodies. The solubility of sulphomucins in the fixatives without alcohol also explained the partially controversial observations previously presented in the literature (*Pearse* 1949, *Marshall & Swettenham* 1959). The presence of sulphomucins in the inclusion bodies has been confirmed by autoradiography, although incorporation of ^{35}S, estimated quantitatively, was low (*Dean & Muir* 1970).

The digestion test using diastase, testicular hyaluronidase and Vibrio Cholera sialidase enzymes described in Table 1 failed to demonstrate any clear effect. *Marshall & Swettenham* (1959) reported a loss of alcianophilia after hyaluronidase digestion and *Muir & Marshall* (1961) demonstrated that the inclusion body material possessed the same electrophoretic mobility as chondroitin sulphate. However, *Dean & Muir* (1970) demonstrated that, although sulphomucin material isolated from Kurloff bodies from spleen tissue resembled chondroitin sulphates, it displayed certain differences. The effect of testicular hyaluronidase which also digests chondroitin-4- and -6-sulphates, remained somewhat unclear although the conclusions drawn by *Pearse* (1949) are in agreement with those drawn on the basis of findings in

the present study namely that we were unable to show any distinct effect of hyaluronidase digestion.

The present study confirms some partially controversial results previously presented in the literature and these results are substantiated by new observations. It is concluded that the material in the Kurloff inclusion bodies contains two types of mucosubstances, both neutral types and sulphomucin(s). The method of preparation used by *Dean & Muir* (1970) failed to demonstrate anything but the polyanionic component which was precipitated by aminoacridine. According to *Marshall & Swettenham* (1959) the histochemical staining characteristics of the sulphomucins in the inclusion bodies resembled for instance, those in cartilage. In the course of the present investigation, however several differences were observed. For one thing, the inclusion body material failed to react with e.g. aldehyde fuchsin and ruthenium red secondly previous periodic acid oxidation is completely absent in the reaction in low and high iron diamine alcianophilia at pH 2.5 finally hyaluronidase digestion has no positive effects. The results of the staining reactions are difficult to express in chemical terms, but they suggest that the density and position of sulphate radicals in inclusion body sulphomucin(s) are different from those in cartilage for instance.

The origin of the inclusion body material cannot be determined on the basis of findings in the present studies. Observation of the ultrastructure, reported in the second part of this study as well as conclusions drawn by *Dean & Muir* (1970) on the basis of autoradiographic studies using ^{35}S incorporation suggest the active secretion of inclusion body material and its storage in the inclusion bodies.

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KURLOFF CELLS

2. Histochemical and Morphological Characteristics of the Kurloff Cells

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Kortelainen, L. & Korhonen, L. K. Kurloff cells. 2 Histochemical and morphological characteristics of the Kurloff cells. Acta path. microbiol scand. Sect. A, 84 154–164 1976.

Kurloff cells (KB cells) containing a glycoprotein inclusion body appear in female guinea pigs and can be induced in males by oestrogen administration. These cells are found in bone marrow thymus, and in the red pulp of the spleen, but not in the lymph nodes. Gradually growing inclusion bodies were observed in a series of cells in these organs. Morphological and histochemical features suggested an active synthesis of the inclusion body material cisternae of the endoplasmic reticulum fused with the membrane surrounding the inclusion body and by way of electron histochemical methods periodate reactive material was demonstrated in the endoplasmic reticulum, in the Golgi cisternae and in the inclusion body Enzymatic activities were not observed in the inclusion body but otherwise the KB cells showed enzymatic activities of oxidative and glycolytic pathways. Marker enzymes for the granulocytes series, the plasma cells the mast cells, and the monocyte-reticulum cell group were negative in the KB cells and thus, classification of the KB cell into a particular cell type was not possible although these cells obviously belong to the lymphoreticular system. Phagocytosis was not observed, either The possible role of the KB cells in the immune system are discussed

Key words Kurloff cell oestrogen effect lymphoreticular system histochemistry electron microscopy

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Although Kurloff cells have been well documented since the end of the last century their origin, nature and function have remained a controversial question due to the rather limited number of investigations in this respect. Kurloff cells are characterized by a large inclusion body they are found under physiological conditions in female guinea pigs, especially during pregnancy and may also be induced in males by oestrogen administration (*Ledingham* 1940) During recent years, it has been suggested that they may play a role in cell mediated immunological processes (*Charles & N[illegible]* 1961 *Christensen et al.* 1970 *Ranlov et al.* 1970 *Marshall et al.* 1971 *Warren et al.* 1971) and accordingly more detailed studies of their characteristics have been motivated.

The present study concerns the histochemical and morphological characteristics of Kurloff cells. In the first part of the investigation (*Kortelainen & Korhonen* [illegible]) the histochemical staining characteristics of the Kurloff inclusion body were reported while the morphological and histochemical characteristics of the cell are discussed in part 2. The investigation was initiated in order to



Fig. 1. A section of the spleen from a guinea pig treated with estrogen over a period of two weeks. Inclusion bodies in the Kurloff cells are seen as black spots. Note the distribution of cells in the zones of macrophages and B-lymphocytes (B) and lacking of them in the zones of T-lymphocytes (T) (Staining PAS-Light Green). × 50

establish whether any marker properties exist which might suggest the origin of the Kurloff cells.

MATERIAL AND METHODS

Male guinea pigs selected as test animals were treated as reported in part I of this study (*Kortelainen & Kerkonen*). Histological samples and samples required for electron microscopy were collected and treated as reported previously.

Besides the staining methods and histochemical reactions described in part I, the following tests were performed: Haemoglobin pseudoperoxidase using a diaminobenzidine method according to Karnovsky (see *Pearse* 1972), dehydrogenases and cytochrome oxidase according to *Pearse* (1972). Cells from blood, bone marrow and stem cells were identified by using histochemical enzymatic reactions according to the instructions given by *Yam et al.* (1971).

With [illegible] demonstrating the periodate reactive material in the electron microscope the methods of *Bradbury & Stoward* (1967) was applied using a 30 min oxidation period, fluorophenylhydrazine serving as substrate.

Phagocytosis was studied by means of colloidal carbon (0.2 mg/100 g) injected intravenously as well as by intravenous injection of Thorotrast® (1 ml/animal). The guinea pigs were killed two days after these injections.

RESULTS

The Kurloff cells appeared in peripheral blood, bone marrow and spleen as well as in the thymus tissue a few days (approximately 4 to 6) after a single injection of 0.5–1 mg of oestrogen applied to adult male guinea pigs. The number of cells was related to the amount of oestrogen. It could be increased after subsequent injections. They increased in number until 10 to 14 days after a single injection and decreased during the following weeks, but a small number of Kurloff cells were found even 4 or 5 weeks after the oestrogen administration.

In the bone marrow, small groups or

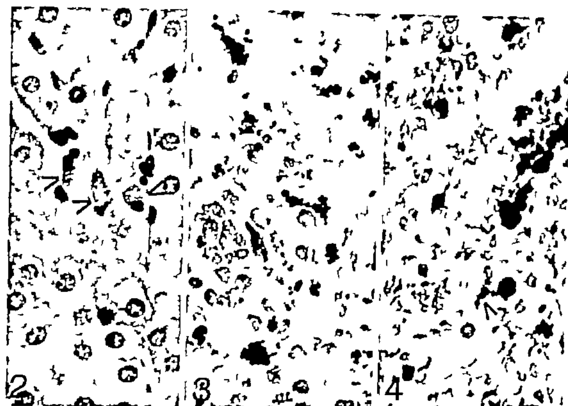


Fig 2 Kurloff cells (arrows) in a section of the liver from a guinea pig treated with oestrogen over a period of two weeks. These Kurloff cells are present in the sinuses of the liver. It is impossible to decide whether they are situated like reticulo-endothelial cells of the liver or whether they were carried to the sinuses by the blood stream. (PAS-HE) × 600.

Fig 3 A section of the spleen from a guinea pig treated with oestrogen over a period of two weeks. This animal received also an intravenous injection of carbon. Numerous macrophages containing phagocytized carbon particles can be seen, but Kurloff cells have not phagocytosed the carbon × 50. (PAS)

Fig 4 Haemosiderin is visualized inside numerous macrophages and in some cells containing a Kurloff inclusion body (arrow) × 500.

single cells were randomly scattered among the haemopoetic cells and were always found in the sinuses of bone marrow. In the spleen Kurloff cells were seen in the red pulp, but not in the lymphoid tissue of the white pulp (Fig 1). Most of the Kurloff cells were associated with splenic cords, some being closely related to the collagen bundles, others lying apparently freely within the venous sinuses. Kurloff cells in the thymus *were most numerous at the cortico-medullary* junction and might be scattered in small clusters in the cortical part of the organ. Only a few cells were observed *in the me*dullary areas of the thymus. Subcapsular collections of Kurloff cells were also found in addition to those around the vessels in the fat tissue surrounding the thymus. Some cells were observed in the lymphatics and blood vessels of the thymic tissue. It was especially noted that Kurloff cells were not seen in lymph nodes except if they were abundant also in the peripheral blood, spleen, thymus and bone marrow. Cells in lymph nodes located in blood vessels and capillaries and *only occasionally in the sinuses*. Blood vessels in other organs also displayed Kurloff cells, e.g. they were seen in the sinusoids of the liver (*Fig 2*).

Typical Kurloff cells were easily identified



Fig. 5. Electron micrograph of part of Kurloff cell showing microfibers (arrow) in the cytoplasm. I = inclusion body × 60 000.

Fig. 6. Margin of the inclusion body of the Kurloff cell seen in Fig. 5 myelin figures are seen. × 76 000

on the basis of a large inclusion body which gave a strong PAS-reaction and was azurophilic in Giemsa stain. Besides these cells, others containing a smaller inclusion body and occasionally cells containing two or three small inclusions, were also seen. These were interpreted as "young" cells or cells which were producing inclusion bodies. Under the light microscope these cells closely resembled macrophages they were found in the spleen and, especially in bone marrow

In plastic-embedded semithin sections stained with alkaline Toluidine Blue, Kurloff cells were easily identified by the deeply stained inclusion bodies. In electron micrographs, the typical Kurloff cell was of a relatively smooth and rounded shape only a small number of microvilli projected from the surface. They displayed the usual cell organelles rough-surfaced endoplasmic reticulum and free ribosomes, mitochondria and Golgi apparatus. Vesicles of variable sizes and filled with fine, granular material appeared in the cytoplasm. The nuclei were generally ovoid or indented, bounded by a nuclear envelope in which nuclear pores were evident. The nuclear chromatin was generally marginated, forming irregular clumps, and one nucleolus was observed In the cytoplasm, bundles of microfibres were often seen (Fig 5 and Figs. 4 5, 6 in part 1)

The inclusion body occupied most of the cell cytoplasm of the typical Kurloff cells. If tissues were fixed in phosphate-buffered glutar-aldehyde the inclusion body would be completely electron-dense but if cacodylate buffer were used it would be more translucent (see part 1) It was surrounded by a single membrane, and the peripheral areas displayed several myelin figures. Often, the

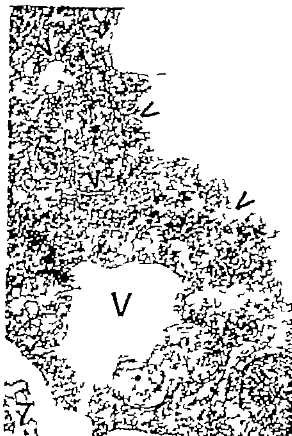




Fig 7 Electron micrograph of part of a Kurloff cell showing extensions (arrow) from the inclusion body to the cisternae of the endoplasmic reticulum. V = dilated vesicels connected with the ER × 40 000.

Fig 8 Periodate-reactive material in an inclusion body (*I*) with projections into the cytoplasm. *N* = nucleus of the Kurloff cell. × 8 000

Fig 9 Periodate-reactive material in the Golgi region of a Kurloff cell. × 24 000

marginal parts were irregular and small projections of inclusion body material extended into the cytoplasm. These extensions seemed to be intimately connected with the endoplasmic reticulum (Fig 6, 7) except for some myelin figures in the peripheral areas of the inclusion body no internal structures were observed.

If the method of *Bradbury & Stoward* (1967) was used to demonstrate the periodate reactive material in electron microscopy the massive inclusion body in the Kurloff cells would give a strong positive reaction. In addition, periodate reactive material was seen in the Golgi apparatus as well as filling of some of the cisternae of the endoplasmic reticulum. Projections of the same material were continuing from the ER to the inclusion. These findings suggest that a synthesis of inclusion body material in the rough endoplasmic reticulum and an intracellular transport of material is required to form the Kurloff inclusion body (Fig 8, 9)

Some cells with an inclusion body comparable with that in typical Kurloff cells but with features similar to macrophages were observed in the spleen (Fig 10–12) In these cells, the most striking features were the numerous processes on the surface of the cells and the general irregularity in shape Vacuolar systems were also observed they were interpreted as multivesicular bodies and



Fig. 10. Electron micrograph of part of a spleen from a guinea pig which had received an injection of Thorotrast. The Kurloff cell is seen to contain a typical inclusion body (I), a flattened nucleus and phagocytosed Thorotrast particles (T) in the cytoplasm. Notice the pseudopods, typical of macrophages. × 18 800. (Fixation: phosphate buffered glutaraldehyde)

phagosomes at various stages of development. Signs of phagocytosis or increased endocytosis were not seen in the typical Kurloff cells, except for a few myelin figures in the cytoplasm, and some degenerating mitochondria. Since cells resembling macrophages were rare the question remains whether or not such cells were true macrophages which had phagocytosed the Kurloff cell material, or whether they might be macrophages which were developing into typical Kurloff cells. A continuous series by which to illustrate the latter hypothesis was not observed.

The phagocytic capacity of Kurloff cells was tested in guinea pigs with traumatic haemorrhage resulting in a considerable accumulation of haemosiderin pigment in the reticuloendothelial system of the spleen. Although numerous macrophages, and reticuloendothelial cells exhibited haemosiderin deposits with the Prussian Blue reaction, haemosiderin was observed only in two or three cells with Kurloff inclusion bodies, out of the two hundred Kurloff cells counted (Fig. 3). Similar phenomena were observed in studies of the distribution of intravenously administered colloidal carbon: macrophages and reticuloendothelial cells were filled with carbon particles, but carbon was only occasionally observed in cells with Kurloff inclusion bodies (Fig. 4). Phagocytosis was further tested using intravenous Thorotrast® and electron

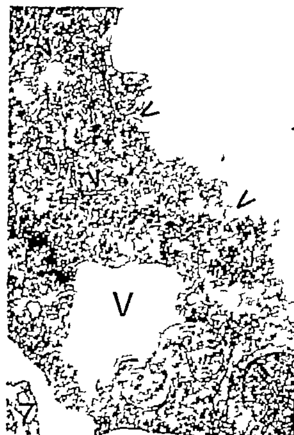






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ABLE 1 *Histochemical Staining Test for Enzymatic Activities in Kurloff Cells and Some Related Cells*

	Nonspecific esterase	Chloroacetate esterase	Peroxidase	Acid phosphatase	Alkaline phosphatase	Metachromas Toluidine Blu
loff cells	0	0	0	0(+/0)	0	+
ocytes	++++	0/+		++++	0	0
culum cells	++++	0/+	0/++	++++	0	0
ma cells	0/+	0	0	0	0	0
phocytes	0/(+)	0	0	0	0	0
t cells	++	+++	0	0	0	+++
trophilic granulocytes	0/++	+++	+++	+++	+++	0
nophilic granulocytes	0/+	0	+++	0	0	0
philic granulocytes	0	0/+	0/++	0	0	++++
loblasts-promyelocytes	0/+	+++	+++	++	0/+	0

these reactions showed enzymatic activities in the cytoplasm of the Kurloff cell, comparable to those observed in the reticulum cells and fibroblasts in the same preparations. The inclusion body was enzymatically inactive. Although the qualitative demonstration of a few enzymes involved in energy metabolism is not conclusive, this observation, and the demonstration of normal cell organelles such as mitochondria, rough surfaced endoplasmic reticulum and free ribosomes in the Kurloff cells, is indicative of active metabolic pathways in the Kurloff cells.

Histochemical staining reactions were also performed using certain other enzymes that could be considered "marker enzymes" by which to distinguish definite cell types of peripheral blood and bone marrow. It appears from Table 1 that Kurloff cells did not seem to belong to any of these particular cell types.

DISCUSSION

Kurloff cells can be induced in guinea pigs by administration of oestrogen or oestrogen derivates. They are found in female guinea pigs, especially during pregnancy. The total quantity of the induced inclusion body material may be as much as 0.5 gram in one spleen, and several grams in one animal (*Nadel* 1952, *Dean & Muir* 1970, *Marshall & Swettenham* 1959, *Welsh* 1966).

The organ principally responsible for the production of Kurloff cells remains obscure. It has been claimed to be the thymus or the spleen (*Bimes* 1964, *Nadel* 1952). Still, thymectomy failed to have any effect on the number of Kurloff cells in the blood (*Ranlov* 1970) and the number was even increased after splenectomy (*Hemle & Heidinger* 1944). In the present study several developmental stages of Kurloff cells were found to be present within the splenic reticulum; the same phenomenon has been reported by *Christensen et al.* (1970). In our preparations series of developmental stages were also obvious in the bone marrow. These findings suggest an active formation of Kurloff cells in the bone marrow and the spleen. At the same time phagocytosing cells in the spleen may remove the Kurloff cells from the blood. The production in other organs (bone marrow) may be extensive to such a degree that the spleen or the thymus can be removed thought the number of Kurloff remains unaffected.

Mitotic figures were not seen in any of the typical Kurloff cells studied during this investigation; the same observation has been reported by *Revell et al.* (1971). Some authors have observed a low mitotic activity (*Marshall & Swettenham* 1959, *Welsh* 1966) but the examples given in their reports suggest that the mitotic figures observed actually represented mitoses in macrophages containing some PAS-positive granules.

Kurloff cells have been considered deriva

tives of lymphocytes (*Jolly & Ferester* 1929 *Bimes et al.* 1964 *Simmons* 1963 1965 *Ledingham* 1940 *Welsh* 1966, *Marshall et al.* 1971 *Nadel* 1952, *Carr* 1970 *Lendrum* 1952) if so it is difficult to explain why they are generally absent in lymph nodes or splenic lymphatic areas (although they are numerous in the red pulp) Furthermore, Kurloff cells are resistant to x rays and to radiomimetic substances (*Nadel* 1952, *Barer & Joseph* 1965 and *Welsh* 1966) This suggests that Kurloff cells are different from typical lymphocytes, despite some superficial similarity in morphological features.

The monocyte macrophage series has been proposed to be the origin of the Kurloff cell *Frey* (1937) *Duplan* (1954) *Barer & Joseph* (1965) Morphological signs of phagocytosis were not revealed by electron microscopy and the experimental use of marker substances in the present study failed to reveal signs of phagocytosis which is in agreement with findings in several previous investigations (*Welsh* 1966, *Berendsen & Telford* 1966 *Revell et al* 1971) The few cells which contained phagocytosed material as well as an inclusion body were presumed to represent macrophages containing material resembling a Kurloff inclusion body or macrophages which actually contained phagocytosed inclusion body material. The histochemical tests for "marker enzymes in the monocyte-macrophage series of cells were negative as regards Kurloff cells, which is in support of the postulation that these cells are different from macrophages. According to the above facts the theory of erythrophagocytosis, proposed by *Barer et al* (1963) is also improbable The results obtained in tests of the pseudoperoxidase reaction were negative and the "pH signature" test (part 1 of this report) showed that the features of erythrocytes and the Kurloff inclusion body material differed widely The lack of "marker" enzymes for lysosomes for instance, and the negative results observed in tests for acid phosphatase activity speak strongly against a phagocytotic origin of the inclusion body

Some authors have proposed that the Kurloff inclusion body might represent a storage of material derived from other sources, (*Nadel et al.* 1952) or material comparable with that collected in cells in cases of storage diseases (*Mittwoch* 1961) These theories are hardly correct, since active phagocytosis cannot be observed, and because injected extracts of spleens containing Kurloff cells failed to produce Kurloff cells in the recipients (*Marshall & Swettenham* 1959) Besides, according to *Dean & Muir* (1970) the biochemical properties of the inclusion body material do not resemble those of the material found in cells typical of storage diseases the main difference is the large size of the molecules of the Kurloff inclusion body material as compared with the relatively small, degradated molecules found in the cells in cases of storage diseases.

Morphological evidence, of an active synthesis of inclusion body material in the Kurloff cells was obtained in the present study The cell is well equipped with organelles commonly associated with protein and carbohydrate synthesis, i.e. ribosomes, abundant rough endoplasmic reticulum and Golgi apparatus. In some preparations, the continuity of the membrane system of the inclusion body with the membranes of the endoplasmic reticulum was also established (Fig 7) The cells are also equipped with enzyme systems and organelles necessary for the production of energy via oxidative and/or glycolytic pathways.

On the basis of the results obtained in the present study we agree with the conclusion drawn by *Revell et al.* (1971) according to whom, the Kurloff cell cannot for the time being be classified as belonging to any particular cell type, although it obviously belongs to the lymphoreticular system. In this connection it should be noted that oestrogens exert a stimulating effect on the reticulo-endothelial system (*Nicol et al.* 1958) on phagocytosis (*Warren et al* 1971) and on the production of immunoglobulins (*Charles & Nicol* 1961) and that resistance to infections has been found to be lowered in the oestrogen treated animals (*Toivanen* 1966)

Several observations are suggestive of an active synthesis of the inclusion body material in Kurloff cells, although direct evidence is still lacking. Some properties of the inclusion body material suggest that it may be of immunological importance, particularly as regards the cell mediated immunological responses (*Christensen et al.* 1970 *Raulov et al* 1970 *Marshall et al.* 1971 *Warren et al.* 1971) It is peculiar that the Kurloff cells are present only in the guinea pig, on the consideration that principal differences in the immunological processes in this and other mammalian species are not known. *Marshall et al* 1971) however reported the extraction of a protein-polysaccharide, biologically identical to Kurloff body material, from the thymus and spleen of pregnant women, rats and mice. The Kurloff body in the guinea pig may thus represent a peculiar collection of material that may be present in other animals, though in a more diffuse form, and may consequently represent a model to be used in future studies of this interesting system.

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NUCLEAR SIZE CLASSES IN THE FOLLICULAR EPITHELIUM OF LYMPHOID THYROIDITIS

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Nilsson, G. Nuclear size classes in the follicular epithelium of lymphoid thyroiditis. Acta path. microbiol. scand. Sect. A, 84 165–171 1976

Nuclear size classes of the type originally described by Jacobj were studied in the follicular epithelium of biopsy smears from lymphoid thyroiditis. Thus, a great number of large nuclei in size classes distinct from the bulk of fairly identically sized nuclei could be clearly distinguished. By cytophotometric measurement of Feulgen-DNA, these large nuclei were found to correspond closely to nuclei in separate DNA-content-classes and they were therefore called HDC-nuclei (*High DNA-quantity Class nuclei*). Such nuclei proved to be more common in lymphoid thyroiditis than in normal thyroids and non-toxic goitres without lymphoid thyroiditis. Among lymphoid goitres, the percentage of HDC-nuclei was positively correlated with the age of the patients. In the present paper the relationship between Askanazy cells and HDC-nuclei is also discussed.

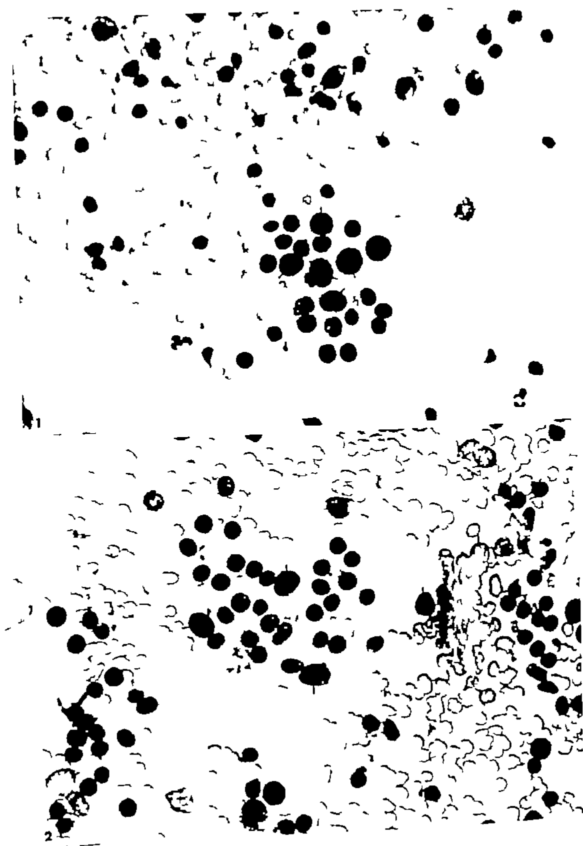
Key words: Thyroiditis, lymphoid; follicular epithelium; nuclear size.

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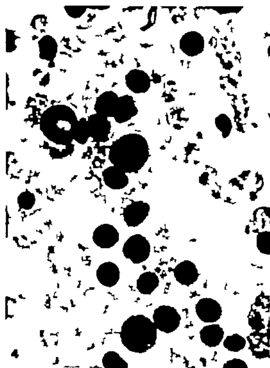
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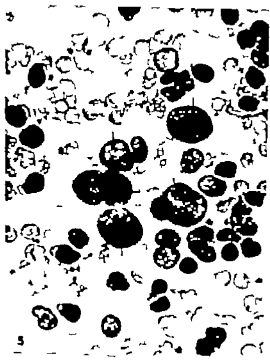
Large cell nuclei are a common striking finding in the follicular epithelium of lymphoid goitres. By examination of fine needle aspirate smears from such goitres, the present author has got the impression that some of these large nuclei fall into separate size-classes clearly differing from that of the bulk of fairly uniformly sized nuclei (Fig. 1 5). This impression of discontinuous anisokaryosis is more pronounced in aspirate smears than in histological sections because the flattening of nuclei in smears exaggerates the nuclear size differences seen in the microscope.

Size classes of the above mentioned type were first described by *Jacobj* (11, 12) who studied the phenomenon in autopsy specimens of several organs, including human thyroid. He also observed that the average volume of nuclear size classes in an organ tended to form a geometrical progression (1.2.4 ...) analogous to the steps of nuclear DNA content later found in non-malignant tissues (18, 20). In thyroid aspirate smears, the Jakobj phenomenon has previously been studied in toxic goitres (15). In this type of goitre a close correspondence between large nuclei in separate size classes and nuclei in separate DNA-content classes was found, the mean DNA-content of which approximately formed multiples (1.2.4 ...) of the mean DNA-content in the basic class. The latter nuclei were called HDC-nuclei (*High DNA quantity Class nuclei*) and they were found to be easily identifiable by estimation of the


1
2







Figs. 1–5 Nuclear size classes in groups of follicular epithelium interspersed between lymphoid cells. Fine needle aspirates from lymphoid thyroiditis. Fig. 1–2 × 160. Fig. 3–5 × 256. Arrows indicate the HDC-nuclei.

size class of the individual nuclei. HDC-nuclei are often called polyploids—a term which is not correct in this connection because such nuclei in terms of the cell cycle (9) may also be diploid cells in the premitotic G2 phase.

The presence of HDC-nuclei in lymphoid goitres is suggested by the previously mentioned large nuclei in separate size classes. These nuclei are of special interest with regard to the Askanazy cell changes which are regarded as a very prominent micromorphological trait of lymphoid thyroiditis. In addition to certain cytoplasmic traits such as a peculiar granulation and special staining characteristics, these cells are described as having especially large nuclei. For reference see *Lindsay* (13) and *Persson* (17).

The present paper concerns a numerical analysis of HDC-nuclei in lymphoid thyroiditis, compared with normal thyroids and non-toxic non lymphoid goitres. It also includes analysis of the occurrence of HDC-nuclei in lymphoid thyroiditis in different ages. This is of interest because of the age dependence of this type of nuclei earlier found in the liver (5).

MATERIAL AND METHODS

Smears with sufficient cellular yield from 27 consecutive cases of lymphoid thyroiditis subjected to fine needle aspiration biopsy were selected for the investigation.

The men:women ratio was 3:24. In all cases diagnosis of lymphoid thyroiditis was confirmed by cytological criteria. Thyroid auto-antibodies against thyroglobulin and/or cytoplasmic antigen (tanned red cell agglutination test and complement fixation test, respectively) were demonstrated in 20 cases of the series thus examined. Three patients were hypothyroid, as judged from clinical examination as well as low serum protein-bound iodine (PBI) and *in vitro* tri-iodothyronine uptake (T_3 test). In the remaining cases, clinical examination invariably revealed an euthyroid state. This was confirmed by the normal values obtained by the PBI and T_3-test, except in three cases in which a slight elevation of the serum protein-bound iodine was found (8.9, 7.9 and 7.8 µg/100 ml; normal range 4.0–7.3 µg/100 ml).

The series of atoxic non-lymphoid goitres and normal thyroids consisted of 20 and 6 cases, respectively. The biopsy specimens in each of these groups were obtained from consecutive cases subjected to fine needle aspiration biopsies. Clinical findings as well as laboratory examinations, including PBI and T_3, invariably argued for euthyroidism in these cases. The non-toxic non-lymphoid goitres included both diffuse and nodular goitres. The thyroids considered to be normal were barely palpable and the biopsies were performed because of obscure pain in the neck or an inexplicably raised erythrocyte sedimentation rate.

The fine needle aspiration biopsies were performed in the way described by *Söderström* (19). The smears were air-dried and stained according to the May-Grünwald-Giemsa technique.

In three cases the nuclear size class estimation was compared with the results of cytophotometric measurements of the Feulgen-DNA-content in individual nuclei. Subsequently, smears made on Bürker-slides were dried in the air and fixed in

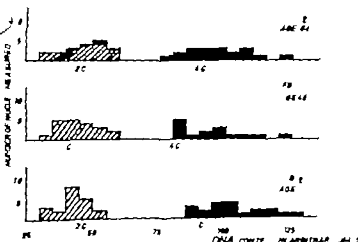


Fig. 6. DNA value of twenty consecutive nuclei judged as HDC-nuclei (black area) and twenty consecutive nuclei judged as non-HDC-nuclei (hatched area) by nucleus size estimation in the follicular epithelium in each of three cases of lymphoid thyroiditis. 2 C = diploid value of DNA.

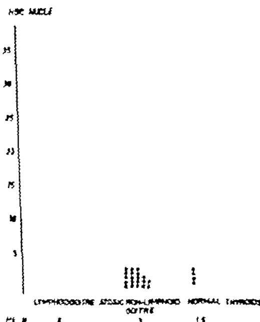


Fig 7 Frequency of HDC-nuclei in the follicular epithelium of three types of thyroid glands. $P < 0.001$ for difference between lymphoid goitres and non-toxic non-lymphoid goitres. $P < 0.001$ for difference between lymphoid goitres and normal thyroids.

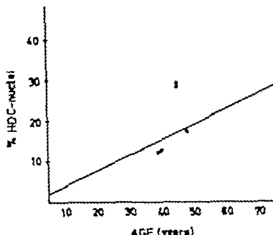


Fig 8 Variation of relative number of HDC-nuclei in the follicular epithelium with age in 27 cases of lymphoid thyroiditis. $y = 0.34x + 2.03$ $r = 0.57$ $p < 0.01$

absolute alcohol for 40 minutes. Hydrolysis was done in 1N HCL for 8 minutes at 60° C. After Schiff staining cytophotometric measurements were made by means of a high resolution rapid scanning microspectrophotometer (3). The DNA-content of individual nuclei was determined at a wavelength of 546 A. The smooth rounded outline of the follicular epithelial nuclei made them distinguishable from lymphoid cells in Feulgen-stained smears. Twenty-five polymorphnuclear leucocytes of the same smears were used as reference for the 2 C value (= "diploid" amount of DNA) after their stain content had been increased by 10 per cent. This was done because human polymorphnuclear leucocytes are reported to have an approximately 10 per cent lower Feulgen-DNA-content than other somatic, non-polyploid, non-DNA-synthesizing cells (7). Feulgen-DNA could not be measured in the majority of follicular epithelial nuclei in the smears because the nuclei tended to lie too close together. In the periphery of the cell groups, however, nuclei were often found in positions sufficiently isolated to allow cytophotometric measurement. From each specimen, twenty consecutive nuclei from the follicular epithelium, judged as HDC-nuclei, and twenty consecutive nuclei, judged as non-HDC-nuclei by nuclear size estimation, were examined for their DNA-content. The results are given in Fig. 6. As seen in this figure, nuclear size class estimation corresponded close to nuclear DNA-content classes. Only in one of 120 nuclei examined there was disagreement between the classification of a nucleus according to size class and DNA-content class. Thus, nuclear size class estimation was considered useful for identification of HDC-nuclei in the present investigation.

RESULTS

The percentage of HDC-nuclei in lymphoid thyroiditis varied widely (Fig 7). In some cases as many as one third of the nuclei were HDC-nuclei—a finding which provided a strong impression of discontinuous anisokaryosis to the cytological specimen. The frequency of HDC-nuclei in lymphoid goitres was significantly larger than that of normal thyroids ($p < 0.001$) and non toxic non-lymphoid goitres ($p < 0.001$). The cytoplasm of the cells with HDC nuclei revealed no trait by which to distinguish them from other cells. As seen in Fig. 8 the frequency of HDC-nuclei was positively correlated with age ($p < 0.001$). Marked discontinuous anisokaryosis is thus seldom found in the juvenile form of lymphoid goitres.

DISCUSSION

The nature of HDC-nuclei in different types of tissues are poorly understood. We can hypothetically propose several formation mechanisms, but reliable supportive data are scanty. It has been observed in rat liver (2, 14) that the rise in the frequency of "polyploids" (defined in the same way as HDC-nuclei in the present investigation) found during physiological growth of the liver as well as during regeneration after partial hepatectomy is associated with a temporary increase in the percentage of binucleated cells. At the same time, mitotic figures of an appearance suggesting nuclear fusion in binucleated cells during the mitotic phase were observed (14). Binucleated cells are rather often found in aspirate smears from lymphoid thyroiditis though the often poorly demarcated cell boundaries in such smears make a quantitative estimation of the frequency of such cells difficult. Thus, it is imaginable that HDC-nuclei in lymphoid thyroiditis may have something to do with a tendency of cytokinesis to fail in the follicular epithelium—a tendency which results in formation of binucleated cells, and subsequent fusion of the nuclei in, for instance, a common mitotic phase. However, other formation mechanisms of HDC-nuclei must also be considered such as higher rate of regeneration of tetraploid than of diploid nuclei or a prolonged premitotic G_2-phase of the type described by *Gelfant* (6) and *Pedersen* & *Gelfant* (16). No data are available for an evaluation of these possibilities in lymphoid goitres.

In animal experiments, HDC-nuclei have been found to increase during thyroid stimulation with thiocarbamides and thyrotropin (1). In lymphoid thyroiditis, destruction of the follicular epithelium often results in lowered secretion of calorigenic hormones and consequently increased blood thyrotropin level via the feed-back system between the pituitary and the thyroid. By analogy with the above mentioned results in animal experiments, an increase in blood thyrotropin may be of importance for the larger number of HDC-nuclei found in lymphoid goitres.

The finding of an increased number of HDC-nuclei with age has an interesting parallelism in analogous findings in the normal human liver (3) as well as in rat liver (2, 14). Thus, a general pattern of increased fraction of HDC-nuclei with age must be suspected and studies of other organs in this respect would be of great interest.

The *Askanazy cell* (4) also known as Hürthle cells (10) and oncocytes (8) is a central concept familiar to every cytologist or pathologist interested in thyroid disease. The nuclei of Askanazy cells are described as especially large or characterized by an especially wide variation in nuclear size. The cytoplasm is said to be homogenous and eosinophil and sometimes granulated. For references see *Lindsay* (13) and *Persson* (17). These descriptions seem not quite specific, but typical cytological features are often not easily depicted in words. Most examiners probably have a clear idea of the appearance of Askanazy cells and there is probably a good correspondence in the identification of these cells by different examiners. The present writer is of the opinion that one important feature of the Askanazy cells is the HDC-nucleus. It is also noteworthy that Askanazy cells and HDC-nuclei are especially common in lymphoid thyroiditis and that the increased frequency of HDC-nuclei with age in lymphoid thyroiditis corresponds to a similar relationship between age and Askanazy cells (17).

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CORRELATION OF ENZYME HISTOCHEMICAL AND STRUCTURAL SEGMENTATION IN THE PROXIMAL CONVOLUTED TUBULE OF THE RAT KIDNEY

Enzyme Activity Compared to the Freeze Dried Structure of Serial-Sectioned Normal Rat Nephrons

TOVE NØRGAARD

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Nørgaard, Tove, Correlation of enzyme histochemical and structural segmentation in the proximal convoluted tubule of the rat kidney Enzyme activity compared to the freeze-dried structure of serial-sectioned normal rat nephrons. Acta path. microbiol. scand. Sect. A, 84 172 182, 1976.

In the normal rat kidney enzyme histochemical activity was correlated with the structural segmentation of the convoluted part of the proximal tubule, as seen in freeze-dried sections. Serial sections were employed for alternate morphological and enzyme histochemical studies. The tubules were investigated for activity of the following enzymes 1) non-specific acid phosphatases, 2) non-specific alkaline phosphatases, 3) succinate dehydrogenase and 4) non-specific esterases. Close to the urinary pole *acid phosphatase* activity was slight in all instances, whilst in the first and second segment a gradual increase in tubular cells with heavy enzyme activity was seen. All tubular cells at the urinary pole showed heavy *alkaline phosphatase* activity but there was a gradual increase of cells showing slight enzyme activity in the first and second segments. *Succinate dehydrogenase* activity was constantly heavy at the urinary pole, and there was a gradual decrease in these cells with heavy enzyme activity along the course of the first and second segments. The pattern of tubular enzyme activity for these three enzymes was independent of the nephron level in the renal cortex. The *non specific esterase* activity was, in comparison, uniform throughout the length of the proximal tubule in nephrons from all levels of the renal cortex. This combined enzyme histochemical and morphological investigation demonstrates conclusively that there is a close correlation between structural segmentation and the pattern of enzyme activity of non-specific acid and alkaline phosphatases and succinate dehydrogenase in the proximal convoluted tubule of normal rat kidney

Key words Kidney tubules proximal tubular segmentation freeze-drying histocytochemistry kidney enzymes.

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Characteristic structural differences in cells from the different segments of the proximal convoluted tubule have been reported before, particularly in ultrastructural studies (*Maunsbach* 1966) Similarly differences in enzyme

activity in different parts of the proximal convoluted tubule have been demonstrated (e.g. *Jacobsen et al.* 1967). The aim of the present study has been to correlate the two parameters: structural segmentation of the proximal tubule as seen in freeze-dried tissue, and the varying activity of different enzymes throughout the length of the tubule.

MATERIALS AND METHODS

Kidney used for alternate enzymatic and morphological investigations. Six normal, white male rats, weight 250–300 g and about 14 weeks old were anaesthetized by intraperitoneal injections of sodium pentobarbital, 100 mg per kg body weight. A left sided nephrectomy was performed, and the kidney was instantly frozen in isopentane cooled by liquid nitrogen to about –165 °C (*Faarup et al.* 1971). 4 µm thick serial sections were cut tangentially to the surface of the kidney on a cryostat and each was numbered. A series of sections was taken from three zones in the renal cortex for each enzyme system under investigation: a) superficially, b) intermediate and c) juxtamedullarly. Each series comprised 4 sections which were alternately used for morphological and enzyme histochemical investigations. *Preparation for morphological investigation.* The frozen sections were freeze-dried at –20 °C placed close to osmic acid crystals in a desiccator (*Faarup* 1965) and were thus stained by the osmic acid vapour during dehydration. These sections were finally mounted in paraffin oil. *Preparation for enzyme histochemical investigation.* In the different cortical zones the following enzymes were investigated: 1) non-specific acid phosphatases, 2) non-specific alkaline phosphatases, 3) succinate dehydrogenase and 4) non-specific esterases. The frozen sections for the enzyme histochemical preparations were placed directly on glass slides without previous fixation (*Jensen* 1973) and put into the incubation medium. The incubation medium for non-specific acid phosphatases (*Barka & Anderson* 1963) contained α-naphthylphosphate (Sigma N 7000) as substrate and pararosaniline (Allied Chemical, National Aniline Div. C. I. 42500) as indicator. pH was 6.0 and the temperature about 21 °C. The incubation time was 15 and 25 minutes respectively. The incubation medium for non-specific alkaline phosphatases (*Burstone* 1958) contained naphthol AS-BI phosphate (Sigma N 2250) as the substrate. The indicator was Fast Red Violet LB (Sigma, F 1625). pH was 8.3 and the temperature about 21 °C. The incubation time 2 and 5 minutes respectively. The incubation medium for non-specific esterases (*Barka & Anderson* 1963) contained α-naphthylacetate substrate (Sigma, N 8750) pararosaniline as indicator (Allied Chemical, National Aniline Div., C. I. 42500). pH was 7.2–7.4, temperature about 21 °C and incubation times 10 and 60 seconds. In the incubation medium for succinate dehydrogenase (*Thomas & Pearse* 1961) the substrate was disodiumsuccinate (Fluka, AG 14170) and the indicator nitro-blue tetrazolium (NBT) (Sigma, N 6876). pH was 7.4 and the temperature 37 °C. Incubation time 30 and 60 minutes respectively. After several experiments using different incubation times the scheme mentioned above was chosen, because it offered the most exact localization of enzyme activity and clearcut reactions in the different cells of the proximal tubule. The sections were mounted in buffered glycerolgelatine. As a control similar sections were incubated without a substrate. From each kidney additional sections were stained with H. E.



Fig. 1. Freeze-dried section frozen 1½ sec after the intravenous injection of methylene blue. The dye is located in the glomerulus (GL) as well as intraluminally in the first segment and in the transitional zone of the proximal convoluted tubule (A: urinary pole, B: more distal in segment 1 and C: transitional zone). No dye is found in the lumen of segment 2 (D). Inset: schematic drawing of the segmental sequence of the tubule (× 400).

Acta path. microbiol. scand. Sect. A, 84 172–182, 1976

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Key words: Kidney tubules; proximal tubular segmentation; freeze-drying; histocytochemistry; kidney enzymes.

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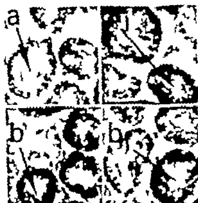


Fig. 3. The range for *slight* acid phosphatase activity (a–a) and *heavy* activity (b–b) as used in the estimation of enzyme activity (× 400).

Correlation of the Freeze-Dried Structure and Enzyme Activity of the Different Segments in the Proximal Tubule

Polaroid microphotographs of the nephrons in the adjacent sections facilitated localization of enzyme activity in the structurally different segments of the tubules. Individual tubules seen at microscopy were readily identified and matched with corresponding tubules on the photographs of their neighbouring sections.

In order to identify the sequence of the morphologically different segments of the proximal tubule an intravenous injection (2 ml) of a 5 per cent methylene blue (Mercks, 6040) solution was given in the right jugular vein of 4 rats of the same weight and age as above—and a left-sided nephrectomy was performed about ½, 1, 1½ and 2½ seconds after the injection. Using this method the tubular cells are stained. The method also demonstrates dye in the tubular lumen which has been ultrafiltrated in the glomerulus. The time interval between injection and nephrectomy in the 4 rats was such, that the dye reached different levels of the nephron, and so the sequence of the segmentation could be established by studying the sections from the 4 rats. Serial sections of the renal cortex were made, each series comprising 15–20 sections. The sections were freeze-dried. The structural segmentation of the proximal tubule was easily seen in the sections and correlated to the presence of dye in the tubular lumen.

RESULTS

The structure of the convoluted proximal tubule in the freeze-dried tissue. In the freeze-dried sections two cell types which were of a structurally different appearance were clearly distinguished in the proximal convoluted tubule: a) tubular cells with a uniform fine granular cytoplasm and without

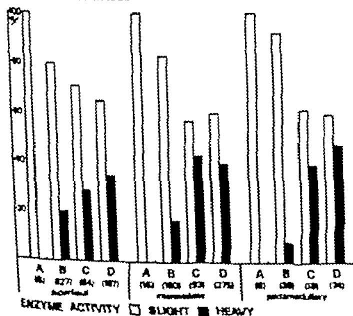


Fig. 4. Diagram showing the percentages of the different segments with slight or heavy acid phosphatase activity in superficial, intermediate and juxtamedullary nephrons. A, at the urinary pole; B, more distal in segment 1; C, transitional zone and D, segment 2. A decrease in tubular cells with a slight enzyme activity and a corresponding increase in cells with heavy activity is seen throughout the course of the tubule independent of nephron level. The figures in brackets are the number of tubules investigated.



Fig 5 Corresponding sections incubated for alkaline phosphatase (5 a) and freeze-dried (5 b) In the proximal convoluted tubule heavy enzyme activity is apparent in both segments (A D) In the tubule marked D from segment 2 slight activity is present (× 400)

radial striation (segment 1 cells) and b) tubular cells with a distinct radial striation of the cytoplasm without granulation (segment 2 cells) In addition in some tubules the cytoplasm of the cells showed both granulation and radial striation transitional cell type. These morphologically different cell types were consistently found in the three different levels of the renal cortex investigated. In the methylene-blue stained sections the sequence of the segments containing cells of different morphological appearance was evaluated. *At the urinary pole* the proximal tubular cells were always finely granular and in the most proximal part of the convoluted tubule the findings were the same (segment 1) *More distally* the tubular cells showed radial striation, and no granulation of the cells was appearant (segment 2) *Between segment 1 and segment 2* a transitional zone was found containing tubular cells with both a granular and a striated cytoplasm (Fig 1) Differentiation into these three segments was independent of the nephron level in the renal cortex. However in the juxtamedullary zone the structure of the tubular cells could be difficult to evaluate due to dilatation of the lumen and decrease in cell height. These changes were probably artefacts which occurred during processing The number of tubules showing the three types of structure was expressed as a per cent of the total number of cross sections of convoluted proximal tubules counted in the sections in the superficial intermediate and juxtamedullary zone of the kidney cortex. Segment 1 comprised about 26 per cent of the tubules counted, segment 2 about 63 per cent and the transitional zone about 11 per cent (Table I) It appears from this table that the percentage



Fig. 6. The range for *slight* alkaline phosphatase activity (a–a_1) and for *heavy* activity (b–b_1) as used in the estimation of the enzyme activity (× 400).

distribution of segment 1, the transitional zone and segment 2 was independent of the nephron level in the renal cortex, and the percentage distribution was similar in the six kidneys investigated.

Non-specific acid phosphatase activity in the proximal tubule. Acid phosphatase activity was a constant feature in the proximal tubular cells and had a granular or spotty appearance and was localized to the basal part of the cells (Fig. 2 a). It was possible to distinguish between two degrees of activity: a) a slight granular reaction termed *slight reaction* (range see Figs. 3 a and 3 a_1) and b) a reaction characterized by coarsely spotted reaction products termed *heavy reaction* (range see Figs. 3 b and 3 b_1). At the urinary pole the enzyme reaction was always slight. In the more distal part of segment 1 most of the tubules showed only slight enzyme activity, but 8 to 20 per cent of the tubules showed a heavy reaction. In the intermediate zone and in segment 2 most tubules showed a slight enzyme reaction, as a heavy reaction was only seen in 29 to 43 and 35 to 47 per cent of the tubules, respectively. Thus along the course of the proximal convoluted tubule an increasing number of tubular cells showed heavy enzyme activity. This pattern was independent of the nephron level in the renal cortex (Fig. 4). *Non-specific alkaline phosphatase activity in the convoluted proximal tubule.* Alkaline phosphatase activity was constantly present in the cells of the proximal

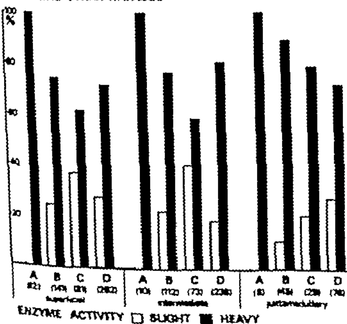


Fig. 7. Diagram showing the percentage values of the segments having slight or heavy alkaline phosphatase activity in superficial, intermediate and juxtamedullary nephrons. A: at the urinary pole, B: more distal in segment 1, C: transitional zone and D: segment 2. At the urinary pole the activity was always heavy. More distal in the proximal convoluted tubule slight activity could be seen, independent of the nephron level. The figures in brackets are the number of tubules investigated.

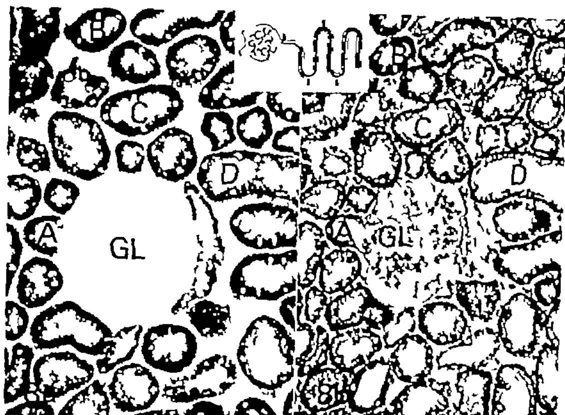


Fig 8. Corresponding succinate dehydrogenase incubated (8 a) and freeze-dried (8 b) sections. The tubules at the urinary pole (A) more distal in segment 1 (B) and in the transitional zone (C) show heavy enzyme activity. Tubule (D) from segment 2 shows slight enzyme activity (\ 400)

convoluted tubule and was localized to the brush border (Fig 5 a) Enzyme activity was again assessed as *slight* (range see Figs. 6 a and 6 a_1) and *heavy* (range see Figs. 6 b and 6 b_1) At the urinary pole the enzyme activity was invariably heavy More distally in the first segment of the proximal tubule 11 to 25 per cent of the tubules had slight activity whilst in the intermediate segments 21 to 41 per cent, and in the second segment 19 to 28 per cent of the tubules showed slight enzyme activity The pattern of enzyme activity in the individual segments of the proximal convoluted tubule was principally the same in the three cortical levels investigated (Fig. 7)

Succinate dehydrogenase activity in the proximal convoluted tubule The cellular enzyme activity showed as fine granules, uniformly scattered throughout the cytoplasm (Fig 8 a) Once more two levels of activity were noted *slight* (range see Figs. 9 a and 9 a_1) and *heavy* (range see Figs. 9 b and 9 b_1) At the urinary pole the activity was always heavy In the more distal part of the first segment 2 to 15 per cent of the tubules showed a slight reaction whilst in the intermediate zone and segment 2 increasingly more of the tubules had a slight reaction, as most of the tubules in segment 2 (62–71 per cent) showed slight enzyme activity The pattern of activity was identical in the three levels of the renal cortex investigated (Fig. 10) *Non specific esterase activity in the convoluted proximal tubule* was granular in appearance and evenly distributed throughout the cytoplasm of the tubular cells (Fig 11 a) The reaction was identical throughout the length of the proximal convoluted tubule, and in each of the three cortical levels stud-



Fig 9 The range for *slight* succinate dehydrogenase activity (a–a_1) and *heavy* activity (b–b_1) as used in the estimation of the enzyme activity (× 400)

ied. Variation of the incubation procedures in no way altered this widespread and uniform distribution of enzyme activity

DISCUSSION

In this study a new method has been applied the correlation of the structural segmentation of the proximal convoluted tubule as seen by light microscopy to the enzyme activity found in the tubular cells of the same segments. In this way a quantitative expression of the variation in enzyme activity along the course of the proximal tubule was obtained. In addition the percentage contribution of the different segments in the proximal convoluted tubule was estimated.

The morphological segmentation of the proximal convoluted tubule Morphological subdivision of the proximal convoluted tubule into two segments (segment 1 and segment 2) was readily seen by light microscopy using stained, freeze-dried tissue (*Faarup et al* 1971) Subdivision of the proximal convoluted tubule has been demonstrated in several ways, *e.g* by phase-contrast and electron microscopy (*Maunsbach* 1966) by light microscopy making use of the varying uptake of dyes in the tubular cells (*Suzuki* 1912) as well as by assessing the varying content of autofluorescent bodies found in the tubular cells (*Sjöstrand* 1944) *Maunsbach* (1966) reported that segment 2 type cells were never found close to the urinary pole and he therefore concluded, that segment 2 constituted the most distal part of the convoluted tubule. In addition, he described tubules with clear

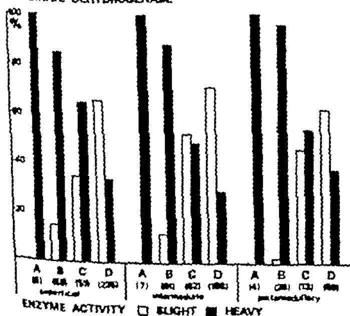


Fig 10 Diagram of the percentage distribution of the tubules with slight and heavy succinate dehydrogenase activity in superficial, intermediate and juxtamedullary nephrons. At the urinary pole (A) the activity was always heavy. More distal in segment 1 (B) through the transitional zone (C) and in segment (D) a decreasing percentage of the tubules shows heavy enzyme activity independent of nephron level. The figures in brackets are the number of tubules investigated.



Fig 11 Corresponding sections incubated for esterase (11 a) and freeze-dried (11 b) No differences in the esterase activity was found in the proximal convoluted tubule. A urinary pol B more distal in segment 1 C transitional zone and D segment 2 (× 400)

acteristics of both segment 1 and segment 2 type cells and concluded, that the transition from segment 1 to segment 2 was gradual. These results tally well with the transitional zone found here in the free-dried sections. Using methylene-blue injected kidneys removed at different time intervals after injection of the dye, the sequence of the segments along the length of the proximal convoluted tubule was readily established, namely segment 1 transitional zone and segment 2.

It appears, that the relative length of the segments was independent of the cortical level. However the morphology in the juxtamedullary region was often very difficult to assess, because of artefact formation. The percentage obtained in the juxtamedullary area is therefore based on observation of fewer nephrons. Nephrons at the juxtamedullary level in the renal cortex have been found to possess a greater glomerular filtration rate than the more superficially placed nephrons (*Rouffignac* 1975) In the present work, however no difference in the percentage distribution of segment 1 and 2 was noted in the three cortical levels.

Correlation of Enzyme Activity and of the Structural Segmentation of the Convoluted Proximal Tubule

Non specific acid phosphatases. Non-specific acid phosphatase activity has been reported as finely granular in appearance (*Jacobsen et al.* 1967) In the present experiments unfixed sections were employed, and this probably resulted in the more spotty enzyme pattern obtained. This was also noted by *Wachstein et al* (1962) who demonstrated that both the enzyme activity and the

pattern are dependent on the treatment of the tissue prior to enzyme incubation. The enzyme activity in the present study was found to correlate well with the structural segmentation of the convoluted proximal tubule (Fig. 4) as segment 1 was found to have a much lower activity than the transitional zone and segment 2. In addition, the proximal tubular cells close to the urinary pole constantly showed only slight enzyme activity. This agrees with the work of *Jacobsen et al.* (1967) who found that the enzyme activity was more pronounced in the distal part of the proximal convoluted tubule than at the urinary pole. They did not, however correlate the enzyme activity to distinct tubular segments. Non-specific acid phosphatase is a lysosome marker and lysosomes have been found to play an important role in protein absorption (*Maunsbach* 1966, *Straus* 1962). In the normal rat heavy acid phosphatase activity thus suggests a high rate of protein absorption in the most distal part of the proximal convoluted tubule. After injection of low molecular proteins (*Oliver* 1948, *Maunsbach* 1966, *Straus* 1964) showed, that the highest rate of protein uptake was located in the distal part of the proximal convoluted tubule. *Straus* (1964) also demonstrated, that the most active nephrons involved with protein uptake were found in the superficial renal cortex. The enzyme activity pattern found in the present study does not correspond to this finding as the percentage distribution of the different enzyme activity was the same at each cortical level.

Alkaline phosphatases. Alkaline phosphatase activity is located in the brush border of the cells and has seldomly been quantitatively estimated in the different segments of the convoluted proximal tubule in previous studies. *Jacobsen et al.* (1967) reported that the enzyme activity was of the same intensity throughout the convoluted tubule. In the present work a clear difference in enzyme activity was constantly noticed between tubular cells at the urinary pole (all cells demonstrating heavy activity) and the tubular cells in the remainder of the proximal convoluted tubule where small numbers showed slight activity (Fig 7). The enzyme pattern of alkaline phosphatases was independent of the nephron level. However *Schmidt & Dubach* (1969) using a spectophotometric method upon microdissected proximal convoluted tubules from the rat cortex found, that the superficially located cortical tubules had a higher alkaline phosphatase activity than that of the juxtamedullary region.

It is well known, that glucose concentration decreases along the course of the convoluted proximal tubule. Alkaline phosphatases may be related to the transtubular transport of glucose (*Schmidt & Dubach* 1969). This present study fits in well with this suggestion, as the activity of the enzyme decreases along the course of the convoluted proximal tubule.

Succinate dehydrogenases. The succinate dehydrogenase activity of the different segments of the convoluted proximal tubule demonstrated quantitatively a very interesting parallel to the structural segmentation. The findings of heavy enzyme activity constantly present at the urinary pole—irrespective of the nephron level investigated—followed by a gradual decrease in heavy activity tubules was only rendered possible by the combined structural and enzyme investigation.

The relation of the enzyme and of the tubular function has been discussed by several authors (*Sternberg et al.* 1956, *Jacobsen & Jorgensen* 1973). Succinate dehydrogenase activity is recognized as a mitochondrial enzyme associated with the oxidative metabolism of the cell. This study shows, that the highest activity is present in the first part of the convoluted proximal tubule, and this therefore indicates, that these cells have a higher metabolic activity than the more distal cells in the convoluted proximal tubule.

Non specific esterases. In this study the uniform activity of this enzyme in cells throughout the convoluted proximal tubule agrees with the results of other investigators (*Wachstein* 1955, *Wachstein & Bradshaw* 1965).

In conclusion in the proximal convoluted

tubule a close enzyme association with particular tubular segments was demonstrated concerning acid and alkaline phosphatases as well as succinate dehydrogenase.

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ATYPICAL FIBROXANTHOMA OF THE SKIN

A Clinico-Pathological Study of 57 Cases

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Dahl, I. Atypical fibroxanthoma of the skin. A clinico-pathological study of 57 cases. Acta path. microbiol. scand. Sect. A, 84: 183–197 1976.

A retrospective study of 57 patients with atypical fibroxanthoma of the skin is presented. The light microscopy is described and the differential diagnosis is discussed. Most of the typical fibroxanthomas (52 out of 57 cases) were originally diagnosed as soft tissue sarcomas, e.g. fibrosarcoma, dermatofibrosarcoma, neurofibrosarcoma, myosarcoma and unspecified sarcoma. The tumour occurred chiefly in middle-aged and elderly patients three patients were 15 years old or younger. The median age was 73 years in patients in whom the tumour occurred in the head and neck, and 34 years in patients in whom the tumour developed on the extremities and trunk. The sex ratio (male to female) was almost equal. Follow-up information about 43 patients was available. The follow-up period ranged from 1 year to 25 years with a median of 9 years. Eight patients died from intercurrent disease all the other 35 patients are alive and well. The clinical course was benign in all but one patient in whom a recurrence developed and metastases to the regional lymph nodes appeared 7 years after the initial excision. The tumour in this case did not differ histologically from the other atypical fibroxanthomas with respect to cellularity cellular and nuclear atypia or mitotic activity. It is suggested that the recurrence *per se* might be of prognostic importance.

Key words: Soft tissue tumour fibrous histiocytoma fibroxanthoma, atypical malignant fibroxanthosarcoma.

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Fibrous histiocytoma is a generic term coined by *Stout & Lattes* (1967) it applies to tumours composed of cells differentiating both into fibroblasts and histiocytes and occurring in various organs. Benign fibrous histiocytomas include fibrous xanthoma (fibroxanthoma) of the skin giant cell tumour of soft tissue (villonodular tenosynovitis) and naevoid histiocytoma (juvenile xanthogranuloma) all of which are easily identifiable histologically (*Stout & Lattes* 1967) Other fibrous histiocytomas may give rise to diagnostic problems, classification and assessment of their biological nature because of their cellularity atypia and varied histological picture.

Atypical fibroxanthoma of the skin, as first described by *Helwig* in 1963 is considered to be closely related to fibrous histiocytoma (fibroxanthoma) of the skin, but shows a greater degree of cellular pleomorphism with mono- and/or multinucleated bizarre cells as well as numerous mitotic figures, some atypical (cf *Enzinger et al* 1969 *Ackerman* 1973) Some 300 cases of atypical fibroxanthoma of the skin are recorded in the literature (*Kempson & McGavran* 1964

Reed 1967 *Kroe & Pitcock* 1969 *Niemi* 1970 *Tapernoux et al* 1971 *Hudson & Winkelmann* 1972, *Fretzin & Helwig* 1973 *Vargas-Cortes et al.* 1973) and these also include lesions referred to as fibrosarcoma (*Halpert & Hackney* 1949) tumours of fibrosarcomatous structure (*Gentele* 1951) sarcomalike tumours (*Rachmaninoff et al.* 1961) paradoxical fibrosarcoma (*Bourne* 1963) pseudosarcomatous dermafibroma (*Levan et al.* 1963) pseudosarcomatous reticulohistiocytoma (*Gordon* 1964) and pseudosarcoma (*Finlay-Jones et al.* 1971) *Gentele's* series of 40 cases of "locally malignant fibroblastic tumours related to noduli cutanei" (1951) may have included atypical fibroxanthomas, but the lesions were difficult to classify on the basis of the descriptions given and hence, they are not discussed here.

Atypical fibroxanthoma of the skin often presents as a small lesion in sun-exposed skin areas of elderly individuals, but may also occur on the limbs and trunk of younger patients (*Fretzin & Helwig* 1973) The clinical course of atypical fibroxanthoma of the skin has almost always been benign. However recurrences have been described (*Gentele* 1951 *Rachmaninoff et al.* 1961 *Bourne* 1963 *Levan et al.* 1963 *Kempson & McGavran* 1964 *Reed* 1967 *Kroe & Pitcock* 1969 *Hudson & Winkelmann* 1972 *Fretzin & Helwig* 1973 *Vargas Cortes et al.* 1973) and 4 cases of metastases have been reported in the literature (*Lattes* 1964 *Caffier & Sorger* 1973 *Fretzin & Helwig* 1973 *Jacobs et al* 1975)

This paper presents the results of a clinicopathological study including follow-up of 57 patients with atypical fibroxanthoma of the skin in one case, the tumour recurred and histologically verified metastases to the regional lymph-nodes appeared 7 years later

MATERIAL AND METHODS

Twenty-two out of the 57 cases in the whole series were taken from a Swedish series comprising some 800 cases of malignant soft tissue tumours reported to the Swedish Cancer Registry during a 6-year period (1958–1963) Twenty-eight cases were selected after reviewing various skin lesions recorded in the Departments of Pathology Sahlgren's Hospital, Gothenburg and the Central Hospital, Vänersborg between the years 1947 and 1969 as sarcoma, fibrosarcoma, dermatofibrosarcoma, neurofibrosarcoma, myosarcoma, dermatofibroma, fibroxanthoma, cellular fibroma, neurofibroma and haemangioma. Seven cases, seen in the period from 1970 and onwards, have been diagnosed as atypical fibroxanthoma of the skin.

Histological Methods

Sections of tissue routinely stained with haematoxylin and eosin and/or the haematoxylin-van Gieson method were available for review in all cases. In 48 cases, sections of paraffin embedded tissue were stained by Masson-trichrome for collagen, Gordon-Sweet for reticulin fibres, van Gieson-elastin for collagen and elastic fibres, the Prussian-blue reaction for iron-containing pigments, the Fontana Masson stain for melanin granules and Sudan-black B for fat. Formalin-fixed, frozen sections, available in 2 cases, were stained for fat.

RESULTS

The sex and age-distribution of the 57 patients with atypical fibroxanthoma of the skin is shown in Fig 1 The youngest patient was 6 years old, the oldest being 89 years old. The median age of patients in the whole series was 37 years. The anatomic location of the tumours is shown in Table 1 Ten of the

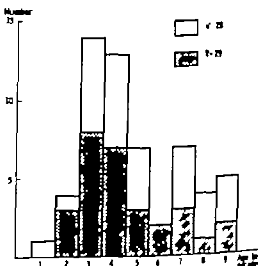


Fig 1 Sex and age distribution atypical fibroxanthoma 57 cases.

TABLE 1 *Atypical Fibroxanthoma, 57 Cases*

Tumour location		No. of cases
Head and neck		11
Upper extremity		22
Upper arm (shoulder)	12	
Fore arm	7	
Hand	3	
Chest and abdomen		4
Back		7
Lower extremity		11
Thigh (hip)	5	
Leg	4	
Foot	2	
Total		55*

Information not available in two patients.

11 patients with atypical fibroxanthoma involving the head and neck area were 60 years old or more. The median age of these 11 patients was 73 years, in contrast to the median age of 54 years of the 44 patients with atypical fibroxanthoma of the trunk and the extremities.

Information concerning the growth rate of the tumours was available in 41 cases. The tumour had been present for less than 6 months in 16 patients, between 6 months and 2 years in 18, and for more than 2 years in 7. In one of these 7 patients, the tumour had been slowly growing for almost 8 years and in another for 10 years. Ten patients reported that growth of the tumour had accelerated during the last months prior to operation. Mild pain or tenderness was experienced by 4 patients. A definite history of trauma prior to the appearance of the tumour was given by 2 patients. It was not known whether any of the sites where the tumours subsequently appeared had ever been exposed to radiation.

Gross Appearance

Macroscopically the tumours were flat or slightly elevated (Fig 2 and 4) in most cases. In 4 cases the centre of the tumour was umbilicated and in 9 cases the tumour was elevated and pedunculated. The cut surface was gray to gray-white in all cases and tinged with yellow or light-brown in 11 cases. The widest diameter of the tumour was 1 cm or less in 30 cases, between 1 and 2 cm in 24 cases, and larger than 2 cm in 3 cases. The largest tumour in the series was approximately 3.5 cm in diameter.

Microscopic Findings

All the tumours were located entirely or almost entirely in the corium. Twenty-eight tumours extended to varying depths of the subcutaneous tissue (Fig 4), deeply in 5 cases, and reaching the adjacent muscle fascia in 2 (Fig 3). In one case, the tumour was located in the external ear and extended to the cartilage. The tumours were separated from the overlying epidermis by a zone of preserved corium in 25 cases. In the additional 32 cases, the tumours abutted on the epidermis (Fig 2) which was ulcerated in 19 cases (Fig 3). In 5 of the ulcerated tumours, the surface was covered by a capillary-rich granulation tissue (Fig. 4). Forty-eight tumours were diffusely growing and poorly delimited (Fig. 2). The additional 9 tumours were slightly nodular and rather well-circumscribed with compression of the adjacent tissue. No tumour was encapsulated.

All the tumours were cellular exhibiting anaplastic cytological features. The tumour cells were principally of two types, one fibroblast and the other histiocyte-like. The fibroblast like cells were elongated, spindle-shaped with elongated, slightly irregular dark nuclei with pointed ends (Fig 5 and 14). In some areas, these cells were rich in eosinophilic cytoplasm and the nucleus was larger, oval, vesicular and showed chromatin clumps or nucleoli (Fig 6). These cells were arranged mostly in a fascicular pattern with interlacing bundles and a suggestion of whorls. The histiocyte like cells (Fig 9 and 10) were richer in eosinophilic cytoplasm which in some cells was homogeneous (Fig. 11) and in other cells slightly foamy or vacuolated (Fig 12). The nucleus was larger than in the fibroblast-like cells, round or oval, vesicular and showed an irregular nuclear membrane and prominent eosinophilic nucleoli (Fig 9). These cells

were arranged more haphazardly (Fig 9) without any distinctive organization. Mono- and multinucleated large pleomorphic tumour cells with abundant eosinophilic homogeneous or foamy cytoplasm and one or more bizarre, hyperchromatic nuclei were frequently found in 21 tumours (Fig 12 and 13)

The fascicular pattern predominated in 37 tumours and the histiocytic pattern in 10. Ten tumours showed a mixture of the two patterns.

All tumours showed mitoses (Fig 7) but the mitotic frequency varied within and between the tumours. In 27 tumours, the frequency was estimated to be one or more in every high power field (HPF × 400). In 19 tumours, the mitotic rate was approximately one in every 5 HPF and in the additional 11 tumours, the mitotic rate was one in every 10 HPF. Atypical mitotic figures were observed in 11 tumours (Fig 8). All tumours were rich in capillaries and vascular spaces with extravasated erythrocytes or small haemorrhages were seen in 15 tumours. Dilated capillaries were seen peripherally and subepidermally in 8 tumours. Ten tumours displayed signs of blood resorption and deposition of haemosiderin in varying amounts. Small areas of necrosis were observed in 5 tumours. Collagen and reticulin fibres surrounded tumour cells, in places running parallel to spindle-shaped fibroblast like cells. Elastic fibres were seen in the fascicular areas between the spindle-shaped cells. Tumours composed of more haphazardly arranged histiocyte like cells were devoid of, or contained only small amounts of elastic fibres, the reticulin fibres were sparse and delicate often surrounding small groups of cells. Lymphocytes and occasional plasma cells were often grouped at the periphery but in some areas, the inflammatory cells mingled more diffusely with the tumour cells. Only small quantities of melanin granules were seen near the epidermis or adjacent to an ulceration never within the tumour tissue. The formalin fixed, frozen sections available from 2 tumours showed variable amounts of intracellular lipid in the histiocyte-like cells. The Sudan-black B stain on paraffin sections revealed small quantities of intracellular lipid in many tumour cells. Intracytoplasmic cross striation or longitudinal fibrils were not demonstrated in the tumour cells.

Primary Diagnoses

In 32 out of the 50 retrospectively collected cases the primary histological diagnosis was sarcoma. Eleven had earlier been diagnosed as fibrosarcoma, 13 as dermatofibrosarcoma protuberans, 2 as neurofibrosarcoma, 3 as leiomyo- or myosarcoma and 3 cases as unspecified sarcoma. In a further 7 cases, malignancy and a high risk of recurrence was suggested. Six tumours were considered to be highly cellular but probably benign, and the remaining 5 were considered to be of benign nature.

Treatment and Follow-up

Fifty-four of the patients were treated by local excision. Wide surgical excision of the lesion was performed initially in 3 patients. Wide excision was performed immediately after the initial biopsy in 18 patients. In 2 of these patients, skin grafts were necessary to close the wound. Amputation of a toe was performed in 1 patient and amputation of the whole external ear in 2. Regional lymph node-excisions were performed in 2 patients.

Fig 2 Elevated atypical fibroxanthoma located entirely in the corium. The tumour is predominantly fascicular diffusely growing, the tumour cells being mingling with the collagen (black) t the periphery of the corium. Haematoxylin-van Gieson × 5

Fig 3 Ulcerated predominantly fascicular atypical fibroxanthoma extending deep into the subcutaneous tissue reaching adjacent muscle fascia. Haematoxylin-van Gieson. × 5.

Fig 4 Atypical fibroxanthoma of mixed pattern. The superficial part is predominantly histiocytic the surface being covered by a thin layer of granulation tissue. The deeper part is fascicular extending into the adjacent subcutaneous tissue. Haematoxylin-van Gieson. × 5


2


3


4

One patient received *radiotherapy after local* excision of the tumour

In 43 cases, follow-up information was available the 7 cases which were diagnosed in the period from *1970* and onwards plus 36 out of the 50 retrospectively selected cases. The follow up period ranged from 1 year to 25 years with a median of 9 years. Six out of the 7 cases diagnosed in the period from 1970 and onwards were alive after 1 to 5 years one patient died from intercurrent illness 1 year after treatment. Twenty nine out of the 36 retrospectively selected patients are alive and well, the length of the follow up period in these cases ranged from 5 to 25 *years, with a median of 13 years. The addi*tional 7 patients in this group died from intercurrent disease 2 to 13 years after treatment the survival time averaging 7 years.

In one patient, the tumour recurred and eventually metastasized. This patient was a 29-year-old woman who in July 1964 was exposed to surgery on account of a small tumour on her left arm she had been aware of the tumour for almost 1 year The tumour was excised in pieces and 7½ months later a small nodule appeared in the scar The tumour and the scar *were removed by local* excision (Fig 15) The primary and the recurrent tumours (Fig 16 and 17) showed almost the same histological picture and were interpreted as atypical fibroxanthomas of the skin. Subsequently the patient remained well for 6 years, but in July 1971 she presented *multiple round tumours up to the size of a* walnut in the left axilla. All of the tumours and fat tissue in the axilla was excised and the area was irradiated post-operatively *(5 000 rad) The surgical specimen showed* tumour growth in the fat tissue as well as in the lymph nodes (Fig 18) Histologically the tumour cells were predominantly spindle shaped *fibroblast like intermingled by histio*cyte-like cells (Fig. 19) The tumour growth was interpreted as metastatic growth from the earlier operated and recurrent atypical fibroxanthomas of the skin Since *then* the patient has been under observation and repeated X ray examinations of the chest have *showed normal* conditions. By now in April 1975 the patient is well without any signs of recurrences or metastases.

DISCUSSION

The tumours discussed in the present study were cellular with anaplastic nuclear features *and varying numbers of mitoses. The cellular* pattern varied both within and between the tumours areas with plump, spindle-shaped *cells in a fascicular pattern were observed* as well as areas with more pleomorphic histiocyte like cells haphazardly arranged. These cellular features and the arrangement of the tumour cells seem to agree *well with the find*ings obtained in a series of atypical fibroxanthoma of the skin described by *Fretzin & Helwig* (1973)

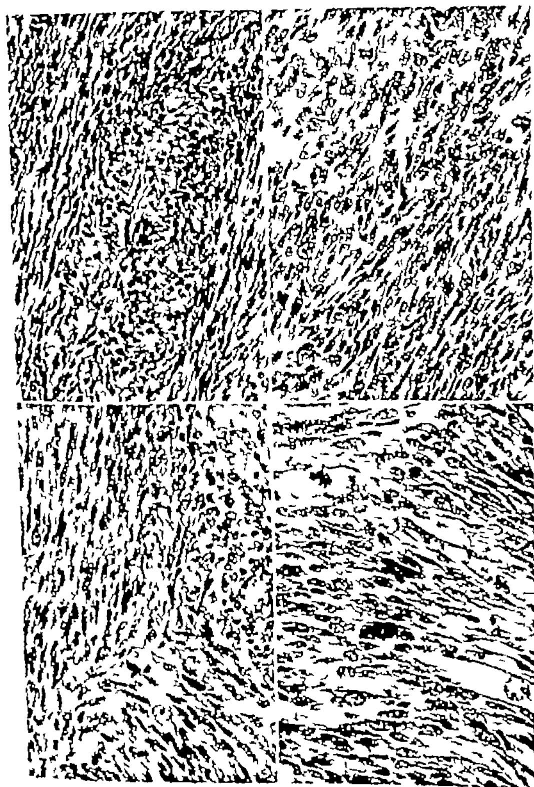
The tumours were distinguishable from fibroxanthoma (dermatofibroma, histiocytoma) of the skin by their greater degree of cellularity and more prominent cellular and nuclear pleomorphism as well as the greater mitotic frequency In *Niemi*'s (1970) survey of 379 cases of "dermatofibroma" and "histiocytoma" of the skin, mitoses were observed in *only 2 cases. Furthermore, spontaneous* ulceration, seldom seen in fibroxanthoma of the skin (*Niemi* 1970) was seen in approx

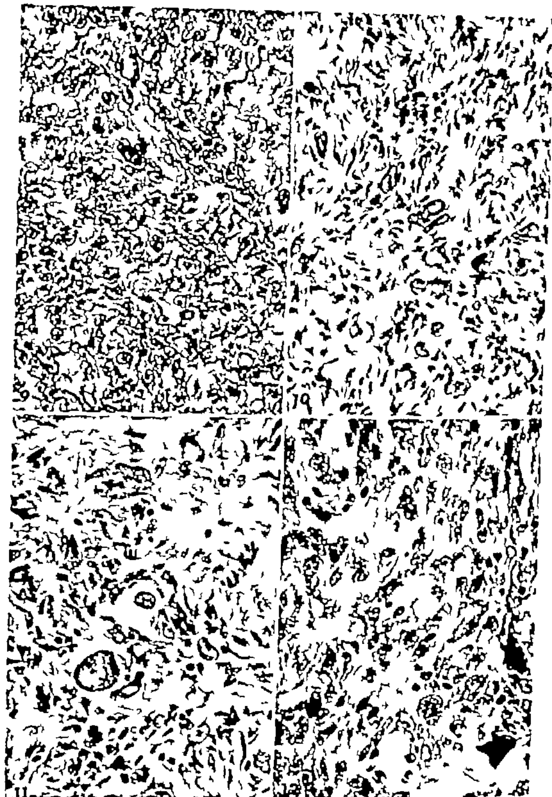
Fig 5 Fascicular pattern of typical fibroxanthoma. The tumour cells are spindle-shaped fibroblast like, *containing elongated nuclei with pointed ends.* Haematoxylin-eosin. × 250.

Fig 6 Atypical fibroxanthoma of predominantly fascicular pattern. The tumour cells are elongated *and the nuclei are large, oval, often* vesicular with chromatin clumps or nucleoli. Haematoxylin-van Gieson. × 400

Fig 7 Atypical fibroxanthoma where the tumour cell are arranged in bundles. The tumour cells are pleomorphic with nuclear and cellular atypia and presenting many mitoses. Haematoxylin-van Gieson. × 400

Fig 8 Cellular and pleomorphic areas in an atypical fibroxanthoma. The tumour cells are large and bizarre mono- or multinucleated. Note the atypical mitotic figures. Haematoxylin-eosin × 400





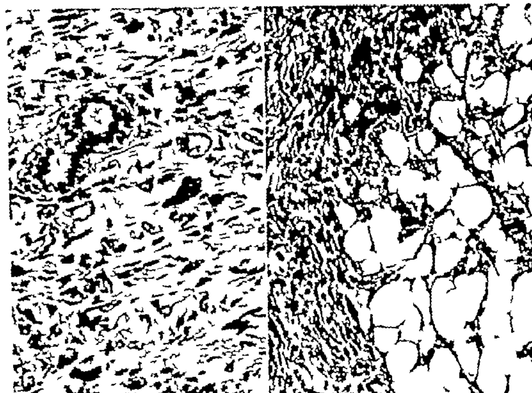


Fig. 13 Atypical fibroxanthoma showing pleomorphic histiocyte-like cells surrounding preserved sweat glands in the corium. Haematoxylin-eosin. × 400.

Fig. 14 Atypical fibroxanthoma in which spindle-shaped fibroblast-like tumour cells extend diffusely into the subcutaneous tissue surrounding single or small groups of fat cells. Haematoxylin-van Gieson. × 250

Fig. 9 Atypical fibroxanthoma presenting a histiocytic pattern. The tumour cells are rich in cytoplasm and are haphazardly arranged. The nuclei are large, irregular and vesicular with prominent nucleoli. Haematoxylin-eosin. × 400.

Fig. 10 Intermingling of elongated spindle-shaped fibroblast-like cells and larger atypical histiocyte like cells in an atypical fibroxanthoma of mixed type. Haematoxylin-van Gieson. × 250

Fig. 11 Atypical fibroxanthoma presenting a predominantly histiocytic pattern. The tumour cells are large and bizarre with pleomorphic nuclei. In the centre, a tumour cell rich in homogeneous eosinophilic cytoplasm. Haematoxylin-eosin. × 400

Fig. 12 Atypical fibroxanthoma showing large bizarre tumour cell with monstrous nuclei. The cytoplasm appears vacuolated and foamy in places. Haematoxylin-van Gieson. × 400.

imately ½ of the cases in the present series.

Atypical fibroxanthoma of the skin has been considered to be an inflammatory reactive process associated with actinically damaged skin and/or previous trauma or irradiation of the area. It applies to 20 per cent of all atypical fibroxanthomas of the skin reported in the literature that they have been associated with previous trauma and irradiation (Table 2). However it is obvious from the present study that atypical fibroxanthomas often occur in non-actinically damaged skin areas or in areas which have not been subjected to trauma or irradiation.

In the differential diagnosis, several lesions have to be taken into account. The tumour has been seen to be associated with nodular fasciitis (*Levan et al* 1963 *Bourne* 1963



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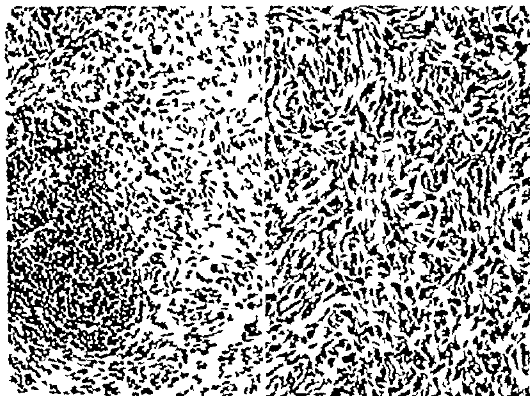


Fig. 18. Metastasizing atypical fibroxanthoma. The tumour cells grew in preserved lymph node tissue (lower-left). Haematoxylin-van Gieson. × 250.

Fig. 19. Metastasis in the axillary region of atypical fibroxanthoma showing principally the same histological picture as the primary and the recurrent tumours depicted in Figs. 16 and 17. Haematoxylin-van Gieson. × 250.

Vargas-Cortes et al. 1973, *Vieira* 1970). It can be difficult to distinguish between nodular fasciitis and atypical fibroxanthoma, but several features may be of help. Nodular fasciitis usually shows myxoid or loosely textured areas with proliferation of spindle-shaped or stellate-shaped fibroblasts and capillaries, often in a radial pattern. Nodular fasciitis is usually subcutaneously situated and involvement of the corium is seen only occasionally (*Allen* 1972, *Dahl et al.* 1972).

Spindle and/or epithelioid cell naevi (juvenile melanoma), as well as malignant melanoma, can cause confusion and must be excluded. Juvenile melanoma occurs mostly in children and adolescents, but can also occur in patients over 20 years of age (*Echevarria & Ackerman* 1967). The presence of adjacent intra-epidermal extension of the tumour cells as well as their nesting or alveolar pattern in malignant melanoma may be helpful in the differential diagnosis. Juvenile melanoma very rarely ulcerates spontaneously and the characteristic small atypical naevus-like

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Fig. 15. Recurrent tumour in the case of metastasizing atypical fibroxanthoma. The tumour extends deeply into the subcutaneous tissue; the adjacent large vessels are not invaded by the tumour. Note the zone of preserved corium between the tumour and the epidermis. Haematoxylin-van Gieson. × 3.

Figs. 16 and 17. The primary and the recurrent tumour in the case of metastasizing atypical fibroxanthoma showing almost the same histological picture. The tumour cells are spindle-shaped fibroblast-like, intermingled with histiocyte-like cells; they are moderately pleomorphic with some large atypical nuclei. Haematoxylin-van Gieson. × 400.

TABLE 2 *Atypical Fibroxanthoma of the Skin 287 Cases*

Author(s)	Number of cases Male	Female	Total	Location Head/neck	Trauma	Radiation	Recurrences
Helpert & Hackney 1949	2		2	2	1		
Gentele 1951	5	6	11	10	1	4	3
Rachmaninoff et al., 1961	2	3	5	3		5	3
Bourne 1963	7	6	13	13	2	1	1
Levan et al. 1963		2	2	2			1
Gordon 1964	4		4	4	1		
Kempson & McGavran 1964	15	6	21	17	3	3	2
Reed 1967	20	3	23	23	3	2	3
Kroe & Pitcock 1969	5	5	10	10		2	1
Niemi 1970	3	1	4	4		3	
Finlay-Jones et al., 1971	7	6	13	12			
Tapernoux et al. 1971	1		1	1	1		
Hudson & Winkelmann 1972	11	8	19	17		11	1
Fretzin & Helwig, 1973	93	47	140*	109	5	6	9
Vargas-Cortes et al. 1973	8	11	19	14		1	4
Total	183	104	287	241	17	38	28

* Two cases previously reported.

cells with lack of cohesion in malignant melanoma were not seen in any of the present cases. Moreover all the tumours in the present series contained only small quantities of coarse and irregular black-brown granules to be demonstrated by the Masson-Fontana stain. The black-brown granules were present in the subepidermal zone of the tumour or adjacent to an ulceration, but unlike features in malignant melanoma, there were no intra or extra-cellularly granules within the major part of the tumour This finding was also reported by *Fretzin & Helwig* (1973)

Owing to the fascicular pattern of spindle shaped fibroblast like cells, the vascular slits and the extravasated erythrocytes, atypical fibroxanthoma of the skin can be confused with fibroblast like lesions in Kaposi's sarcoma. Areas of pleomorphic histiocyte-like cells are not seen in Kaposi's sarcoma and neoplastic proliferation of capillaries (*Reynolds et al* 1965 *Lever* 1967) was not demonstrable in the atypical fibroxanthomas The clinical course of Kaposi's sarcoma with multiple nodules and plaques is fairly characteristic (*Cox & Helwig* 1959 *O'Brien & Brasfield* 1966) atypical fibroxanthoma of the skin may be multiple but, to my knowledge, only two such cases have been published (*Hudson & Winkelmann* 1972)

Differentiation between the so-called "spindle-cell carcinoma" of the skin and atypical fibroxanthoma is considered a great problem (*Reed* 1967 *Hudson & Winkelmann* 1972) Signs of squamous differentiation such as abnormal intracellular keratinization and/or intercellular bridges were absent in all tumours in the present series and a well-defined zone of preserved corium between the tumours and the epidermis was evident in almost half of the tumours

It is not always easy to distinguish between poorly differentiated cutaneous leiomyosarcoma and atypical fibroxanthoma The blunt ended nuclei, often aligned in tandem or in rows, the intracellular longitudinal fibrils as well as the cytoplasmic tinctorophilia in trichrome-stains are the characteristics of leiomyosarcoma that make such a tumour recognizable (*Dahl & Angervall* 1974) However previous studies of cutaneous leiomyosarcoma and the present review show that,

In the case of some spindle cell tumours of the skin, it may be impossible to determine by the light microscope whether they originate from histiocytes or muscle. Such cases are not included in this series.

Embryonal rhabdomyosarcoma in the skin occurs usually on the head or neck (*Albores Saavedra et al.* 1963, *Angervall et al.* 1972). It is possible to distinguish this highly malignant tumour from atypical fibroxanthoma by identifying the round, strip-, ribbon- or racquet-shaped rhabdomyoblasts which are the hallmarks of embryonal rhabdomyosarcoma (*Pack & Eberhart* 1952). Moreover, unlike atypical fibroxanthoma of the skin, this tumour occurs particularly in children.

In many cases, the primary diagnosis was dermatofibrosarcoma protuberans. Dermatofibrosarcoma protuberans may be distinguished from atypical fibroxanthoma of the skin by way of the more uniform appearance of the tumour cells and the storiform pattern of growth (*Enzinger et al.* 1969). The atypical fibroxanthoma with the fascicular pattern showed in places a tendency to form a whorling pattern, but this was not a prominent feature. Dermatofibrosarcoma protuberans is often multinodular (cf. *Taylor & Helwig* 1962) and the irregular and indistinct linear or lamellar extension of the tumour into the subcutaneous tissue as well as the storiform pattern of the tumour cells in dermatofibrosarcoma protuberans was not found in any tumour in the present series.

Dermatofibrosarcoma protuberans may belong to the fibrous histiocytoma group (cf. *Stout & Lattes* 1967, *Ackerman* 1973). The term storiform fibrous xanthoma has also been proposed for this tumour (*O'Brien & Stout* 1961). *Kempson & Kyriakos* (1972) considered storiform fibroxanthoma to be closely related to fibroxanthosarcoma, a malignant variant of fibrous histiocytoma. Fibroxanthosarcoma occurs mostly in the extremities and is located deep in the subcutaneous tissue, frequently associated with secondary involvement of skeletal muscle. The tumour is in most cases large and may also invade the overlying skin (*Soule & Enriquez* 1972); such cases are not included in the present study.

Clinically, atypical fibroxanthoma of the skin generally presents as a small firm nodule less than 2 cm in diameter. However, the tumours can as seen in some cases in this series be larger than 2 cm and attain a diameter of as much as 5–10 cm (*Fretzin & Helwig* 1973, *Kempson & McGavran* 1964). The lesion appears most often as a rapidly growing tumour of a few months duration, as found in this series, but its growth can occasionally extend over years.

The frequency of occurrence of the tumours was almost identical in males and females which is at variance with the previously reported male predominance (cf. Table 2). According to *Kempson & McGavran* (1964) and *Kroe & Pitcock* (1969), atypical fibroxanthoma affects the skin of the head and neck of elderly people. In the present series, however, atypical fibroxanthoma also occurred in younger patients, predominantly on the trunk and extremities. This is in accordance with the age-distribution in relation to location reported by *Fretzin & Helwig* (1973).

Atypical fibroxanthoma seems to be extremely rare in children. Only 3 patients were 15 years old or less: 2 girls were 15 years old and one boy was 6 years old. In *Fretzin's & Helwig's* (1973) series, 2 patients were below the age of 20 years, one of these was a 6 year old boy. In *Kauffman's & Stout's* (1964) series of malignant and questionably malignant fibrous xanthoma in children, 2 cases (cases 21 and 24) may have been atypical fibroxanthomas of the skin.

In patients with atypical fibroxanthoma of the skin the clinical course is almost always benign and recurrence is infrequent (Table 2). Recurrence occurred in only one patient in the present series and that patient also developed metastases to the regional lymph nodes almost 7 years after the initial excision. Malignant fibroxanthoma (fibroxanthosarcoma) of the *skin* seems to be extremely rare and only four published cases are known (*Lattes* 1972, *Caffier & Sorger* 1975, *Fretzin*

& *Helwig* 1973 *Jacobs et al.* 1975) Another two cases (*O'Brien* & *Stout* 1961 case 40, *Kauffman* & *Stout* 1961 case 20) may also have been malignant fibroxanthomas of the skin. The two cases reported by *Gentele* (1951) as tumors of fibrosarcomatous structure in radio-atrophic skin with clinical evidence of metastases has been questioned by *Reed* (1967) according to whom they were spindle cell carcinomas.

The one case described here as well as those already described in the literature satisfactorily demonstrate that atypical fibroxanthomas of the *skin* can behave in a malignant fashion. Histologically the case presented here was not different from the other atypical fibroxanthomas of the skin with regard to cellularity cellular and/or nuclear atypia and mitotic activity In the other 4 cases of metastasizing atypical fibroxanthoma of the skin reported in the literature, blood vessel invasion was observed in one case (*Fretzin* & *Helwig* 1973) and in another (*Lattes* 1972) the recurrent tumour and the metastases were less differentiated than the initial tumour These features were not observed in the present case. It is of interest to note that recurrences developed in all the 4 previously reported metastasizing atypical fibroxanthoma of the skin and that the only tumour in the present series which metastasized also was the one which had recurred. The prognostic importance of recurrences both in deep (*Cantin et al.* 1968, *Moberger et al* 1968) and in superficial (*Dahl* & *Angervall* 1974) malignant soft tissue tumours has been emphasized.

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SPECIFICITY OF HORMONAL RESPONSIVENESS *IN VITRO*

Effect of Sex Steroids on Non malignant Rat Cells Compared with the Effect on Cells from Rat Tumours

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Aspegren K. Specificity of hormonal responsiveness *in vitro* Effect of sex steroids on non-malignant rat cells compared with the effect on cells from rat tumours. Acta path. microbiol. scand. Sect. A, 84 198–200, 1976.

Since mammary tumours and sarcomas in the rat both have been shown to be sensitive to steroid hormones *in vitro* a search for a non-steroid sensitive rat tissue was made. Neonatal cerebellar medulloblasts in the rat did not respond to high concentrations of steroids if cultured as organ culture. The relevance of this finding to the specificity of steroid response assays *in vitro* is discussed.

Key words: Mammary tumours sarcomas medulloblasts rat hormonal responsiveness organ cultures.

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In two previous publications (1 2) the *in vitro* response of DMBA-induced mammary tumours and sarcomas and virus induced sarcomas in the rat towards steroid hormones was studied by two different methods, bearing in mind the value of such *in vitro* methods for a prediction of hormonal responsiveness of human tumours. Effects of the hormonal treatment were found in all types of rat tumours tested. It goes without saying that these effects were not generally of toxic nature, considering the high steroid concentrations used, since different steroids gave different effects on one and the same tumour while a non-hormonal steroid (cholesterol) did not evoke any reaction (1) Sarcomas are not generally considered responsive towards steroid hormones, but have been little investigated in this respect. Since it could be suspected that the rather unphysiological culture procedures *per se* could account for the similarities in response of the two different types of tumour it was judged to be of interest to investigate whether a non malignant rat tissue might be sensitive to steroids if tested similarly *in vitro*

Receptors of steroid hormones are not confined to the breast and the uterus, but can also be found in the kidney the hypothalamus and in fibroblasts (3) The medulloblasts of the neonatal cerebellum are unique in that they proliferate actively for a couple of weeks after birth, contrary to other nerve tissue cells (4) *Since they are not likely to contain* receptors for steroid hormones, they were chosen for the study

MATERIAL AND METHODS

One week old outbred Sprague-Dawley rats yielded the cerebellum tissue. In each experiment, tissue from six or seven rats was pooled.

The organ culture technique has been described in detail elsewhere (2). In brief, twelve explants, approx. 2 × 2 × 2 mm were placed on a millipore-filter on top of a thin piece of cellulose acetate and floated in Trowell's T8 medium in a Carrel D5 flask. One per cent human albumin, antibiotics and steroids (hormone treated flasks) or steroid without only one per cent ethanol (control flasks) were added.

The following hormones were used in the following concentrations:

17-β-oestradiol 20 µg/ml
Testosterone 50 µg/ml
Progesterone 80 µg/ml

These concentrations were identical with the highest ones used in previous works (1, 2).

The explants were cultured in a Carrel flask for 44 hours. They were then transferred into a glass tube with one millilitre of its own medium. 2 µCi of H^3-TdR (methyl-H^3-thymidine, specific activity 1.9 Ci/mM, Schwartz/Mann Inc., Orangeburg) was added and the culture was continued for another 4 hours. The explants were then centrifuged and washed twice with cold trichloro-acetic acid for half an hour. They were finally dissolved in Soluene 350 (Packard Ltd) and the radioactivity of each flask was determined by liquid scintillation counting.

Six different experiments were performed and all three hormones were tested in each of these. Three flasks were set up for each hormone and three served as controls. Hormone effect was expressed as the difference of the mean $\log_{10}$ cpm of the three control tubes and hormone treated tubes. No correction was made for the DNA content of the tubes (2). Prior to statistical treatment, a general (technical) error variance was determined from the triple determinations. The uniformity of these variances was controlled by Bartlett's test. The significance of hormone effects ($\log$ cpm differences between control flasks and hormone flasks) was tested by F-test between

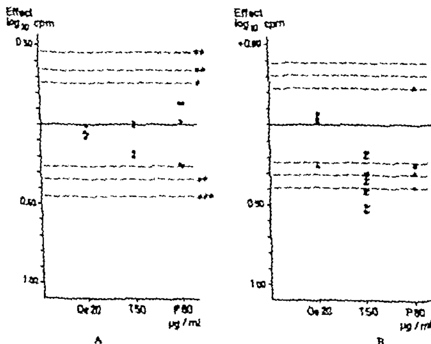


Fig. 1 A. Results of six experiments where rat medulloblasts (dots) simultaneously were organ cultured for 48 hours with 17-β-oestradiol, testosterone or progesterone. Growth in vitro was quantitated by labelling with H^3-TdR for 4 hours. Effect is expressed as mean differences between $\log_{10}$ cpm of control flasks and hormone treated flasks. Dashed lines indicate statistical significance of F-test between variances due to "hormone effect" and "general error". * denotes $0.05 > p > 0.01$, ** $0.01 > p > 0.001$, *** $p < 0.001$.

B. Results of twenty-nine experiments where rat mammary tumours (triangles) and rat sarcomas (circles) were cultured similarly as in Fig. 1 A. The data are derived from a previous work (ref. 2).

variances due to "hormone treatment" and general error

RESULTS

The results are given graphically in Fig 1 A. There was good incorporation of H^3 TdR in all experiments, comparable to the levels observed in previous work of similar nature (2)

Any statistically significant effects of the hormone treatment was not observed in any of the six different experiments in spite of the very large concentrations used. By way of comparison, Fig 1 B shows similarly cultured rat mammary tumours and rat sarcomas. These latter originate from ref 2.

DISCUSSION

As shown previously using this organ culture technique hormone sensitive tumours (DMBA induced mammary tumour) and presumably non-steroid hormone-sensitive tumours (virus or DMBA induced sarcomas) in the rat react similarly to steroids *in vitro* (2) This finding aroused the suspicion that the culture procedure *per se* could influence the reaction of cells to steroids in an unspecific way and thus give toxic effects. But the present series of experiments shows that neonatal cerebellar tissue in the rat is not influenced by the high concentrations of steroid hormones. The result can be interpreted as follows

1) Sarcomas of different aethiology (virus- or chemically induced) are sensitive to steroid hormone. This could be explained by a derepression of genes involved in the steroid receptor synthesis. The possibility seems to have been little investigated if true, it opens up new perspectives in clinical hormone therapy

2) Steroid responsiveness *in vitro* is a property of neoplastic tissues in general and not linked with "specific" steroid responsiveness. If true, predictive *in vitro*-tests by which the influence of steroid hormones on tumour growth may be measured are misleading and may account for the poor *in vitro-in vivo* correlation generally achieved in such studies.

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CYTOPLASMIC EFFECTS OF X-IRRADIATION ON CULTURED CELLS IN A NONDIVIDING STAGE

I. Establishment of an Experimental Model

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The investigation was initiated with the aim of establishing a suitable experimental model with respect to mode of radiation and radiation dose for elucidating morphologically the sequence of development of radiation induced damage of interphase cells (human glia cells in culture). Adequately defined and reproducible cellular changes were obtained using X-radiation generated by an 8 MeV linear accelerator at a dose of 20,000 rad. The cellular alterations were studied in the light microscope and by scanning and transmission electron microscopy. The most conspicuous changes—first appreciable about 5 hours after irradiation—occurred in the lysosomal vacuome and the plasma membrane and associated structures.

Key words: X-irradiation; cytoplasmic effects; cultured cells; experimental model.

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The effects of ionizing radiation on the structure function and viability of different cell types (both normal and neoplastic) have been studied in a variety of systems using a large number of different appr[illegible] These studies have to a great ext[illegible] the attention on the profound inh[illegible] of growth that can be observed in [illegible] exposed to ionizing radiation [illegible] effect of ionizing radiation on cells [illegible] be intimately associated with [illegible] the nuclear DNA *Tolmach* [illegible]

In recent years it has be[illegible] evident that intracellular [illegible] sent another sensitive t[illegible]

radiation (*Harris* 1970, *Myers* [illegible] & *Wiedekamm* 1973). A num[illegible] tigations have been perform[illegible] the exact nature of radiation [illegible] structural alterations in differ[illegible] membrane systems.

A [illegible] difference has [illegible] th[illegible] survival response [illegible] pared to cells in [illegible] A relative rad[illegible] [illegible] cells in [illegible] ming tests of [illegible] used when pr[illegible] by dye exclus[illegible] 1972). It ha[illegible] induced cell [illegible]

ent mechanisms in rapidly growing cells ("reproductive death") and nondividing cells ("interphase death") (*Goldstein & Okada* 1972). Whereas reproductive death is generally thought to result from nuclear damage, cytoplasmic membrane lesions could be of great importance in interphase cell death (*Harris* 1970).

The present paper is the first in a series of studies aiming at elucidating the morphologic effects of radiation damage to interphase cells. When establishing an appropriate experimental model, the use of tissue cultured cells appeared to be necessary.

Glia cells were chosen because of their very well pronounced contact inhibition of mitosis (topoinhibition) which is much more pronounced than in fibroblasts or various types of embryonic cells (*Dulbecco* 1970, *Westermark* 1971). All of the cells in the cultures are thus in approximately the same phase in the cell cycle (G). These cells can be prepared for electron microscopy with good and reproducible preservation of structural details and preserved relationships to each other and the support on which they grow (*Brunk et al.* 1971). These cells are therefore well suited for studies on cytoplasmic effects of irradiation on interphase cells.

The specific aim of the present investigation was to establish the proper experimental model with respect to mode of radiation and radiation dose for studying the sequential development of radiation induced damage of interphase cells. Furthermore, in view of the vast and in many respects contradictory literature on the morphologic effects of irradiation, it was considered important to critically review earlier studies in the field in order to create a base line for the reports to follow.

MATERIALS AND METHODS

Cell Lin and Culture Conditions

All experiments were performed on *in vitro* cultured diploid human glia cells in phase II. The same pool of frozen cells was used throughout the study. The cells were derived and maintained in culture as described by *Pontén et al.* (1969). They were grown in Eagle's minimal essential medium (EMEM) supplemented with 10 per cent calf serum and antibiotics (100 U/ml of penicillin, 50 μg/ml of streptomycin and 1.25 μg/ml of amphotericin B). The cells were harvested for irradiation 5 days after subcultivation when they had just formed a confluent monolayer.

Conditions of Irradiation

Different sources of irradiation were tried.

(*a*) Co^{60} gamma rays supplied by a conventional radiotherapeutic outfit. The dose rate was approximately 200 rad/min.

(*b*) X-radiation, with the conditions of irradiation being: 50 kV, 20 mA, 0.25 mm Al HVD 5.5 mm H_2O. The dose rate was calculated to be 5,200 rad/minute at the cell level.

( ) X-radiation generated by 8 MeV electrons in a Linac accelerator at a dose rate of approximately 1 700 rad/min.

During irradiation, cultures were kept outside the incubator in normal indoor-environment. Controls were kept outside the incubator for as long a period of time as the irradiated cultures and were subjected to the same transport procedures. Doses administered in the different experiments ranged from 500 to 50 000 rad. Cells were harvested for morphologic examination immediately after completion of irradiation and at intervals varying from 30 min to 14 days post irradiation.

Light Microscopy

For light microscopy the cells were fixed in 1/1 mixture of phosphate buffered saline (PBS) and methanol subsequently substituted by pure methanol. The cells were then stained according to Giemsa.

Transmission Electron Microscopy (TEM)

Subsequent to fixation for 60 min at +4 C in 2 per cent glutaraldehyde in 0.1 M Na-cacodylate-HCl buffer with 0.1 M sucrose (pH 7.2, total osmolality = 510 mOsmol, vehicle osmolality = 300 mOsmol), the cells were postfixed in 2 per cent OsO in s-collidine buffer (pH 7.2) for 90 min at 22 C. A short rinse in 0.15 M cac buffer (pH 7.2) at 22 C was interposed between the two fixations. The cells were dehydrated *in situ* in the plastic dishes in a graded series of ethanol solutions, starting with 50 per cent.

The cells were "stained" with 2 per cent uranyl acetate for 10 min in the last change of 100 per cent ethanol. The monolayer was then cut with razor blade into squares about 2 by 2 mm and was removed from the plastic using dissolution with propylene oxide according to *Biberfeld* (1968). The squares were subsequently washed in propylene oxide several times t completely remove all remnants of plastic, and were embedded in

Epon 812. The cell material floating in Epon was centrifuged down in Beem® capsules at [illegible]0 000 × g for 60 min at room temperature. This procedure is slightly modified from a previous detailed description by *Brunk et al.* (1971). Ultrathin sections were cut on an LKB Ultrotome, stained with lead citrate (*Reynolds* 1963) and examined in a Jeol-100C electron microscope.

Scanning Electron Microscopy (SEM)

Cells were cultured on glass coverslips (diameter 12 mm) or specially cut glass pieces, 12 × 6 × 1 mm, placed in plastic Petri dishes. The cells were fixed in the aforementioned glutaraldehyde fixative by replacing the culture medium with warmed fixative (37° C, 5 ml). Care was taken not to dry the cells during the replacement of medium with fixative solution. The cells were postfixed in buffered OsO in the same way as the cultures intended for TEM. Dehydration was performed in a graded series (50 70 75 80 85 90 95 100 per cent) of ethanol. The cells were then brought to acetone and critical-point dried from CO in a Polaron E 3000 apparatus. After mounting on stubs with silver conductive paint, the specimens were vacuum coated with carbon and gold in an Edwards E 12 E 2 evaporation unit. The specimens were rotated and tilted by means of a home-made motor-driven apparatus to allow coating from all angles. They were studied in a Jeol JSM S1 microscope operated at 10 KV or in a Jeol-100C electron microscope equipped with side entrance goniometer and a scanning attachment at 40 KV.

RESULTS

Selection of Radiation Sources

Rather high doses of irradiation were demanded to produce noticeable changes in the contact inhibited human glia cells within the chosen time period. Thus, no definitive changes were observed in cells receiving less than 5 000 rad. The Co^{60} outfit as a radiation-source was therefore unsuitable because of the extremely long irradiation time needed resulting in development of unspecific cellular changes. These changes were mainly dependent on the fact that the cells had to be kept outside the incubator too long.

The conventional X-ray apparatus provided a sufficient dose rate. The cellular damage produced by this kind of irradiation was, however, insignificant and not completely reproducible. This was probably due to the fact that the radiation had a very limited penetration. Accordingly this approach was not pursued.

The dose rate obtained with the X-irradiation generated by the 8 MeV linear accelerator was found to be acceptable, and the results were readily reproducible. Hence, this radiation source was well suited for the purpose of the present investigation, and all observations referred to below have been made on cells subjected to this kind of irradiation.

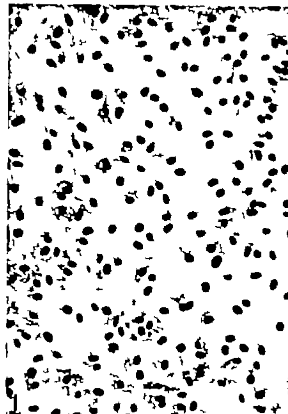
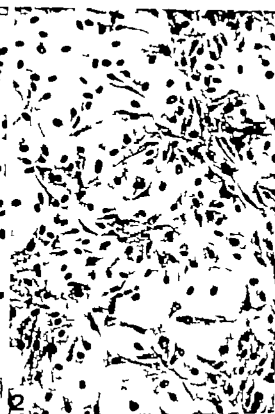
Light Microscopy of Control Cells

The cultures formed a monolayer composed of glia-like cells with abundant cytoplasm and rounded nuclei containing one or two small nucleoli (Fig. 1). The nuclear chroma-

TABLE 1. *Prevalence of Cells with Condensed Nuclei and Cytoplasm with Long, Slender Projections at Various Intervals Post Irradiation*

Dose received	¼	½	1	2 days	3 days	4 days	7 days	14 days	days post irradiation
5,000 r	0	0	+	+	+	+	+	0	
10,000 r	0	+	+ +	+ +	+ +	+ +	+ + +	–	
[illegible]0,000 r	0	+	+ +	+ +	+ + +	+ + +	+ + +	–	

0 denotes no change.
\+ denotes a few altered cells.
\+ + denotes a moderate number of altered cells.
\+ + + denotes a great proportion (50 per cent or more) of altered cells.
– cultures almost devoid of viable cells.


1

2

3

4

lia was moderately dense and evenly distributed. The cytoplasm contained numerous small granules and had a smooth outline with rounded or polygonal shape. Nuclear overlapping was unfrequent except in small areas where cells piled up in a fashion described earlier (*Westermark* 1971).

Light Microscopy of Irradiated Cells

Definite alterations, typically consisting of cytoplasmic and nuclear condensation, were noted in cells receiving 5 000 rad or more (Figs. 2–4). Many cells also formed slender cytoplasmic projections crossing neighbouring cells. The occurrence of these alterations was clearly dose dependent, being slight and rare with low dosage and increasingly frequent and prominent with higher doses. In cultures receiving 10,000 rad or more, cells were haphazardly distributed and formed tight, irregular conglomerates. The cytoplasmic changes were first noted at approximately 24 hours post irradiation in cells receiving 5,000 rad of irradiation and at 12 hours in cells receiving 10 000 rad or more. With higher doses the changes appeared earlier and became more pronounced, as illustrated in Table 1. Occasional severely damaged and probably nonviable cells were noted from 3 days post irradiation onward in cultures exposed to 5 000 rad. In cultures exposed to 10,000 or 20,000 rad apparently dying cells were seen at 24 hours, and by the ninth day there were rather few living cells left in the cultures exposed to 20,000 rad.

In cultures receiving 5,000 rad normal morphology was gradually restored and 14 days post irradiation there were no differences in cell shape or growth pattern between the irradiated cells and controls. This seemed to be the result of the gradual elimination of all irreversibly damaged cells from the cultures. No mitotic cells were observed following irradiation with 5 000 rad or more and irradiated cultures thus stayed sparse. The cytoplasmic changes in irradiated cells were fully developed within 3–4 days in cultures receiving 20,000 or 30 000 rad. From this time onward, severe unspecific degeneration with cell death and steadily augmented cell loss from the cultures prevailed.

Scanning Electron Microscopy of Control Cell

The glia cells in interphase were flattened and as a rule they grew as a monolayer with only occasional overlapping of nuclei (Fig. 5). The upper cell surfaces were smooth except for a variable number of delicate microvilli and haphazardly distributed small caveolae and pits, probably representing endocytotic invaginations. Blebs or blunt extrusions of any kind were never seen on the cells in the stretched-out non-mitotic state.

Scanning Electron Microscopy of Irradiated Cells

No alterations were found at a dose level significantly below 5 000 rad. In cells receiving 5,000 rad, alterations appeared at the 24 hour interval. Following 10 000 or 20,000 rad of X-irradiation distinct alterations were noted at 12 hours post irradiation. The changes (illustrated in Figs. 6–8) consisted in shrinkage, rounding up of the cells and formation of long slender cytoplasmic projections. The condensed cells were noted to leave delicate trailing retraction fibrils in the

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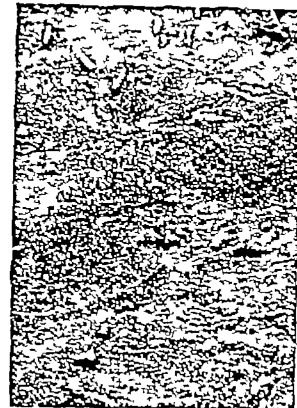
Note. Figs. 1–4 are light micrographs of normal and irradiated glia cell cultures fixed and stained as mentioned in Materials and Methods.

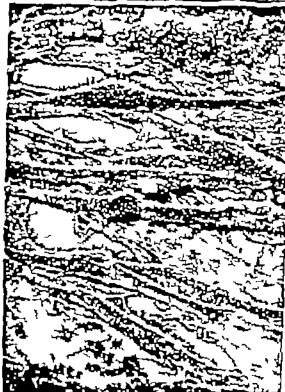
Fig. 1. Control. × 80.

Fig. 2. 24 hours after 20,000 rad. Note irregularity of cellular shape and outline and occurrence of spindle-shaped cells with condensed cytoplasm and nucleus. × 115.

Fig. 3. 72 hours after 20,000 rad. Alterations are similar to those at 24 hours (cf. Fig. 2). However, a much larger proportion of the cells has the slender condensed, spindle-shaped appearance than at the shorter interval. × 115.

Fig. 4. 96 hours after 20 000 rad. High magnification picture illustrating nuclear pleomorphism and pyknosis, occurrence of slender cytoplasmic projections, and general shrinkage and elongation of the cytoplasm. No normal cells are present. 285






areas where cell contact with the Petri dish surface was partially lost.

The growth pattern appeared to be disorganized with overlapping of cytoplasmic projections and of nuclei. Some heavily irradiated cells displayed cytoplasmic blebs all over the surface and in some cells gross vacuolization occurred. Many of the condensed cells showed considerable reduction in the number of microvilli.

Transmission Electron Microscopy of Control Cells

The fine structure of normal contact inhibited glia cells in phase II has been described previously (*Brunk et al.* 1971) The cells in the present study showed a similar appearance They contained few lysosomes and only occasional autophagic vacuoles and residual bodies.

Transmission Electron Microscopy of Irradiated Cells

No definite ultrastructural alterations were noted in cells receiving less than 5,000 rad of X-irradiation. In cells receiving 5,000–10,000 rad, ultrastructural modifications were present from 12 hours post irradiation. During the first 2–3 days the changes mainly consisted in an increased number of autophagic vacuoles containing recognizable cytoplasmic organelles such as mitochondria and endoplasmic reticulum. During the next 2–3 days, a steady increase in the amount of dense bodies of residual body type occurred. The alterations developed earlier and became more pronounced with higher doses. At a dose level of 20 000 rad (Figs. 9–11) the earliest signs of ultrastructural modifications were noted at approximately 5 hours post irradiation, and the residual body formation was fully developed at the 4 day interval. After this time, severe, unspecific degeneration and cell death prevailed.

Exposure of the cells to 5,000 rad or more resulted in formation of blebs and endocytotic vacuoles all over the cell surface. This effect was increasing continuously with dose.

Apart from the aforementioned changes, other cytoplasmic as well as nuclear alterations occurred which partly bore resemblance to the normal "aging" processes observed in the cultured glia cells (*Brunk et al* 1973)

DISCUSSION

Brief Critical Review of the Literature

The possible mechanisms of radiation damage to cell membranes and the consequences of membrane damage to cell function and survival has been reviewed, e.g by *Myers* (1970) and *Wallach & Weidekamm* (1973) The water protein, carbohydrate and lipid contents of the membranes could all be changed through direct as well as indirect radiation effects.

The most important radiochemical mechanisms of membrane damage seem to be oxidation of membrane thiole groups and peroxidation of membrane lipids. Among the intracellular membranes sensitive to SH-oxidation or lipid peroxidation, lysosomal membranes have acquired great interest (see e g *Harris* 1970) This interest is partly based on the fact that lysosomes seem to be highly sensitive to that kind of damage partly on the special implications of lysosomal membrane damage that have been postulated. The lysosomal contents of—for the cytoplasm

Note Figs. 5–8 show the appearance of control and irradiated glia cells in the SEM. For method of preparation, see "Materials and Methods"

Fig. 5 Control. The cells are flat and have numerous villous projections on their upper surface × 950.

Fig. 6 24 hours after 20,000 rad. The cells are elongated, appear contracted ("thicker" than normal) and overlap in some areas. × 1 400.

Fig. 7 48 hours after 20,000 rad. The cells are elongated, spindle-shaped and partly overlapping Certain areas of the surface are devoid of microvilli. × 950.

Fig. 8 96 hours after 20,000 rad. The cell in the center of the picture has rounded up and shows presence of numerous blunt projections and furrows on its surface. Note presence of numerous slender retraction fibrils. × 2,900

AV

9

MP

RB

AV

AV

10

—potentially noxious acid hydrolases and the presumed increase in lysosomal membrane permeability has given rise to the "enzyme release hypothesis" for radiation induced cell damage (*Bacq & Alexander* 1961)

Occurrence of increased numbers of lysosomes and/or enlargment of lysosomes are commonly observed in irradiated cells (*Sobel* 1964, *Lane & Novikoff* 1965 *Brandes et al* 1967 *Ghidoni & Campbell* 1969 *Paris et al* 1969 *Conti et al.* 1974) as are augmented autophagy or formation of residual bodies (*Hugon & Borgers* 1966 a, b, *Masurovsky et al.* 1967 *Paris et al.* 1969 *Rendé et al.* 1971 *Ostenda* 1973 *Conti et al* 1974) Such changes may represent an unspecific response of cells to a number of sublethal injuries (*Ericsson* 1969) In tumors, such unspecific influences include hypoxia and anoxia due to insufficient blood supply to portions of the tumor tissue. Hence, the finding of large numbers of autophagic vacuoles and lysosomes in the cells of irradiated tumors may have been unrelated to irradiation *per se*

Irradiation has been reported to cause an increase in the "activity" of lysosomal enzymes in the cell (*Hugon & Borgers* 1966 a, b *Paris et al* 1969 *Rendé et al* 1971 *Aitkman & Wills* 1974 *Reynolds & Wills* 1974) and apparent increased lysosomal membrane permeability or inferred transfer of cytochemically detectable enzyme activity from lysosomes to cytoplasm has been correlated with subsequent cytoplasmic damage (*Brandes et al.* 1967)

Hypertrophy or increased "activity" of the Golgi apparatus has been observed in different kinds of irradiated cells (*Montgomery et al.* 1964 *Maxwell & Kruger* 1965 *Ostenda* 1973) Histochemically detectable acid phosphatase activity was markedly increased in Golgi saccules of irradiated tumor cells (*Brandes et al* 1967 *Paris et al.* 1969) Other radiation induced alterations are e.g. distension or fragmentation of Golgi membranes (*Sobel* 1964 *Schäfer* 1969)

Multifarious alterations in the endoplasmic reticulum have been described after X irradiation, including hypertrophy proliferation, dilatation, vesiculation, fragmentation, degranulation and recombination of fragments (*Pitcoc* 1962, *Sobel* 1964 *Ghidoni* 1967 *Masurovsky et al* 1967 *Hendee et al.* 1968 *Schäfer* 1969 *Rendé et al* 1971 *Conti et al.* 1974) Many of these alterations, however are also present in tissues subjected to inappropriate fixation.

Different types of mitochondrial damage have also been reported to occur in irradiated cells. Alterations consisted in e.g swelling, vacuolization distortion of contours, occurrence of swollen, villiform or otherwise modified cristae, rupture of limiting membranes, and elongation or branching of the mitochondria (*Montgomery et al* 1964 *Hugon & Borgers* 1966 a, *Ghidoni* 1967 *Masurovsky et al* 1967 *Hendee et al* 1968, *Schäfer* 1969) However complete lack of detectable mitochondrial damage has been reported in some irradiated cells (*Sobel* 1964) Attention has been drawn to the similarity of some of the alterations mentioned above to fixation artifacts in the mitochondria of control tissue (*Jordan et al* 1972)

Apparent damage to the nuclear membrane has been observed in irradiated cells. The perinuclear space in this type of damage is widened, or the nuclear membrane becomes irregular sometimes with formation of myelin bodies (*Pitcoc* 1962, *Montgomery et al* 1964 *Hendee et al* 1968, *Schäfer* 1969 *Conti et al* 1974 *Klein-Szanto et al* 1974) Deep infoldings of the nuclear membrane and nuclear lobulation has been reported

Fig. 9 12 hours after 20,000 rad. Electron micrograph showing two autophagic vacuoles (AV and AV) in the cytoplasm of a glia cell. AV contains cytoplasmic ground substance with no or only minor alterations while the changes in the sequestered portion of cytoplasm are advanced in AV. MP microprecipitate to which the cells are anchored G Golgi regions. × 30,000

Fig. 10 48 hours after 20,000 rad. Picture illustrating fine structure of portions of glia cells, one of which contains numerous residual body-like structures (RB) and two apparent autophagic vacuoles (AV) with alterations in the sequestrated materials × 25,000.



(*Aoyama* 1972, *Klein-Szanto et al.* 1974). Widening of the perinuclear space and occurrence of myelin bodies represent structural modifications which also may occur during the fixation procedure.

Damage to the cell membrane often manifests itself in the formation of "blebs" of different size (*Hendee et al.* 1963, *Montgomery et al.* 1964, *Conti et al.* 1974). Again, such blebs may be formed in the course of improper preparation.

A number of morphologic alterations have thus been reported to occur in irradiated cells. The reports are by no means confirmative; indeed, sometimes they are clearly contradictory. One reason for this lack of consistency may be that most experiments have been performed on normal or neoplastic tissues *in vivo*. Populations of cells in tissues (neoplastic and other) are inhomogeneous with respect to stage in the cell cycle and may vary in their nutritional state—for instance due to differences in blood supply within different areas. It is also difficult to obtain uniform and reproducible fixation of cells in tissues because of variations in the penetration and distribution of the fixative within the tissue. It is also known that the position of a given cell in the cell cycle affects its response to ionizing radiation (*Tolmach et al.* 1971). In most earlier reports, the cells studied (*in vivo* or *in vitro*) seem to have emanated from an inhomogeneous population as far as their place in the cell cycle was concerned. There is presently a lack of agreement concerning the interpretation and significance of the fine structural alterations described in cells of various types of tissues subjected to irradiation. This disagreement may to a large extent be due to the factors mentioned above.

The use of tissue cultured cells would make it possible to circumvent fixation artifacts due to improper penetration of the fixative. Furthermore, all cells in tissue culture are under similar nutritional conditions. Finally, methods exist whereby most of the cells can be brought to the same cell cycle phase.

Design of Present Experimental Model

The experimental system using contact inhibited human glia cells seems to offer a uniquely homogeneous cell population with almost complete contact inhibition of cell division, furthermore, practically all cells are arrested in the same phase (G 1) of the cell cycle (*Pontén et al.* 1969, *Westermark* 1971).

In the system studied, rather high doses of irradiation were required to produce noticeable changes in cell structure. Since the experiments were performed with the aim of elucidating radiation effects on the cytoplasm of contact inhibited cells, no plating experiments were done and no dose-survival curves were obtained. However, doses of the order of 30,000 rad produced no immediate cell death. The relative radio resistance observed was probably related to the fact that the cells were in a state of contact inhibition of cell division. Plateau phase cultures are known to be more radio resistant than exponentially growing ones (*Goldstein* & *Okada* 1972, *Kim et al.* 1973) and the stationary phase of growth seems to favor repair of radiation damage (*Little* 1973, *Evans et al.* 1974) as does close cell contact (*Durand* & *Sutherland* 1972).

For the studies of interphase irradiation damage, 20 000 rad was found to be an appropriate dose. At this dose level, prominent alterations in cellular shape and fine structure developed rapidly enough (v.z. within 1 to 4 days) to minimize the effects of unspecific influences in the culture such as cell aging and changes in the composition of the medium.

Comment on Present Findings

The most conspicuous alterations in the irradiated cells detectable with the light microscope consisted in cellular condensation

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Fig. 11. 96 hours after 20,000 rad. Survey electron micrograph of portion of glia cell containing abundant residual bodies. × 25,000

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coupled with elongation and formation of long slender extrusions. A similar phenomenon has been observed in irradiated rat glial tumor cells within three days after an x ray dose of 300–600 rads (*Heilporn et al.* 1973) and was thought to result from microtubule assembly although the process appeared to be independent of DNA and RNA synthesis.

Outgrowth of axon like processes occurs in cultured mouse neuroblastoma cells exposed to irradiation or dibuturyl cyclic AMP (*Prasad & Vernadakis* 1972) Similar alterations have been produced by other agents and have been thought to represent the morphologic expression of differentiation (*Prasad & Sheppard* 1972 *Prasad et al.* 1973 *Edström et al* 1974) However in our experimental system with well differentiated human glia cells, such alterations could hardly be interpreted as signs of cellular differentiation.

The radiation induced alterations in cell shape observed in our experiments could well be initiated by damage to cell membranes or cell metabolism, leading to a decrease of the cell surface/cell volume ratio. This theory gains support from the SEM findings of condensed cells with fine, trailing retraction fibrils extending from the original points of cell adhesion. Collapsing cytoplasm leaving membranous "streamers attached to the cover glass has been observed e.g. in irradiated cultures of Chang liver cells (*Montgomery et al* 1964) Alterations in intracellular membranes seem to be involved in the earliest phase in the chain of events ultimately resulting in death of the irradiated interphase cell. With high doses of irradiation membranous lesions (in mitochondria and endoplasmic reticulum) have been observed to develop within a few minutes post irradiation (*Hendee & Alders* 1968 *Jordan et al* 1972)

However the exact relation of membrane damage to modifications of DNA or RNA still remains to be elucidated. For instance it is a well known fact that radiation induced death of thymic lymphocytes is associated with a marked loss of the functional integrity of the cell membrane On the other hand, intranuclear depolymerization of RNA has been reported to preceed interphase death of thymocytes (*Geracı et al.* 1974) At least, part of the membrane lesions obtained after irradiation of stationary phase cells can not be excluded to be mediated by some alterations in cellular protein synthesis.

The fine structural observations in the TEM indicated that X-irradiation induced increased autophagy followed by large scale accumulation of residual bodies in the cultured glia cells. This was the most conspicuous fine structural alteration observed, and the finding supports earlier reports. However appearances which may represent preparation artifacts, e.g vacuolization and distortion of mitochondria, fragmentation of the endoplasmic reticulum, or formation of myelin bodies in the perinuclear space were not encountered.

It would therefore seem that the most conspicuous radiation induced alterations in the glia cells occurred in the lysosomal vacuome, and the plasma membrane and associated structures. The development and nature of the changes in these elements will be described in subsequent reports.

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EXPERIMENTAL AVIAN NEPHROPATHY

Changes of Renal Function and Structure Induced by Ochratoxin A-Contaminated Feed

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Krogh, P., Elling, F., Hald, B., Jylling, B., Petersen, V. E., Skadhauge, E. & Svendsen, C. K. Experimental avian nephropathy. Changes of renal function and structure induced by ochratoxin A-contaminated feed. Acta path. microbiol. scand. Sect. A, 84: 215–221, 1976.

One-day-old chickens were fed ochratoxin A-contaminated diets at 2 levels: 0.3 and 1 mg ochratoxin A per kg feed, for 341 days. The only observable lesion to develop was a kidney damage comparable with the naturally occurring avian nephropathy. The changes in renal function were characterized by impairment of glomerular and tubular function, indicated by a decreased inulin clearance, Tm_{PAH} and decreased urine concentrating capacity. The changes of renal structure included degeneration of the tubular epithelium accompanied by regeneration. At slaughter, the kidneys, liver and muscular tissue of the birds contained residues of ochratoxin A (up to 30 µg per kg). As all the birds would have passed the meat inspection because no macroscopical lesions were present, this represents a possible health problem.

Key words: Nephropathy; avian; ochratoxin.

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A recent investigation of hens condemned by the meat inspection due to renal lesions revealed that a high proportion of the birds was suffering from nephropathy characterized by tubular degeneration and interstitial formation of connective tissue (*Elling et al.* 1975). Residues of ochratoxin A were detected in thoracic muscular tissue in all the birds suffering from nephropathy, indicating previous exposure to ochratoxin A-contaminated feed. These findings may suggest a causal relationship of ochratoxin A, a nephrotoxic mycotoxin, to avian nephropathy comparable with the well-documented causal relationship of mycotoxic porcine nephropathy (*Krogh et al.* 1973, *Krogh et al.* 1974). The present study of ochratoxin A-induced changes in renal function and structure was conducted in order to obtain further evidence of ochratoxin A as a disease determinant of avian nephropathy. The exposure levels of ochratoxin A employed in this study (0.3 and 1 mg per kg feed) correspond well with those occurring in naturally contaminated cereals (*Scott et al.* 1972, *Krogh et al.* 1973).

MATERIALS AND METHODS

Experimental Birds

Three groups were studied (group 1 control group 2 and group 3 experimental) each consisting of 27 one-day-old chickens calculated from the time of hatching (*White Leghorn*) The large number of birds per group was employed in order to compensate for losses due to illness and death during this long-term experiment, especially in the initial period. After a growth period of 165 days, the groups were reduced to comprise 7 pullets pr group in order to relate numbers of birds to amount of feed available. The groups were maintained during the egg production period and the experiment was terminated when the pullets had reached an age of 341 days. Egg production and egg quality were measured, and hatchability was monitored based upon 60–70 eggs pr group.

Five additional pullets were fed as the control birds until the last 2 weeks of the experiment when they were included in group 3 (as group 3 a) in order to elucidate short term exposure.

Feed

During the experimental period, the birds had free access to feed and water A lot of barley naturally contaminated with ochratoxin A was incorporated in the diets in order to obtain two levels of ochratoxin A for the experimental groups. During the experimental period, the feed was analysed for ochratoxin (*Nesheim et al.* 1973 and oxalates (*Anon* 1967) Table 1 illustrates the measured concentrations of these two nephrotoxic compounds. The identity of ochratoxin A was confirmed by NMR spectroscopy At the beginning of the experimental period, the feed was found free from two other nephrotoxic metabolites citrinin and viridicatumtoxin, according t *Hald & Krogh* (1973) and *Stey* (1969) and *H tchison et al* (1973) respectively

The daily water consumption, on a group basis, was measured during a 2-weeks period just before the end of the experiment

Renal Functions

At the end of the experiment, the renal function was measured in all birds. A clearance study was carried out, including 3 × 15 min collection of ureteral urine from the hydrated birds under local anaesthesia. The glomerular filtration rate (GFR) was calculated as plasma clearance of inulin. The tubular excretion rate of para-aminohippuric acid (PAH) was calculated as the renal excretion rate after subtraction of the filtered load. In separate *in vitro* ultrafiltration experiments, the PAH concentration of the filtrate was found to be 0 86 of the plasma concentration. After the clearance experiment, the maximal urine osmolality was measured after injection of vasopressin. The level of hydration was controlled by measuring plasma osmolality and total protein concentration. No difference between the groups was observed. A full report of the detailed renal function measurements has been published elsewhere (*Sorensen & Skadhauge* 1976)

Pathology

At slaughter necropsy was carried out on all tissues. Pieces of kidney and liver tissue were fixed, embedded and stained with Haematoxylin-eosin, Iron-haematoxylin-van Gieson and PAS.

Ochratoxin A Residues

Tissues

The remaining part of liver and kidneys, and samples of 50 g of thoracic muscular tissue were analysed for ochratoxin according to a procedure previously published (*Krogh et al.* 1974) Positive findings of residues were confirmed by derivative formation. The procedure has lower level of detection t 2–3 µg per kg.

Egg

Samples of 50 g, drawn on group basis, from well-mixed portions of whole eggs, were extracted with 250 ml chloroform methanol (1:1) with

TABLE 1 *Feed Content of Nephrotoxic Compounds*

	Group		
	1 (Control)	2	3
Ochratoxin A (µg per kg feed)	ND	323 5 ± 62.45	1051 8 ± 123.09
Coefficient of ariation (%)		51.2	31 0
Oxallates (mg per kg feed)	528 ± 41.9	5 1 ± 37 4	631 ± 14.0
Coefficient of ariation (%)	21.0	19.0	5.9

ND = No detectable amount.
= SEM.

added celite 454 in a shaker during 3 hours. After filtration, the liquid was reduced by evaporation to 50 ml. Clean-up was performed according to *Steyn & van der Merwe* (1966). Thin-layer-chromatography was carried out as indicated above. Recovery studies of spiced whole-egg samples revealed 31 per cent recovery at a level of 1.6 μg per kg, and 94 per cent recovery at a level of 3.2 μg per kg.

RESULTS

During the initial period a pronounced mortality due to an attack of avian encephalomyelitis, was encountered in groups 1 (Control) and 3. No deaths occurred and no clinical symptoms were observed during the growth period and the egg production period.

The water consumption, as measured at the end of the experiment, was increased in the experimental groups, especially in group 3 (Fig. 1).

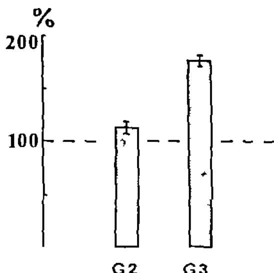


Fig. 1. Water consumption monitored during a 10-days period at the end of the experiment. Group one (control): 74 ml/day/kg bw.

Renal Functions

In the control birds, the glomerular filtration rate (GFR) was found to be 3.03 ± 0.26 ml/kg/min, the urine osmolality being 377 ± 20 m Os, corresponding to an average osmotic urine-to-plasma ratio of 1.74. These values are not reduced as compared with those obtained in previous investigations using similar techniques (*Skadhauge* 1973). The Tm_{PAH} showed saturation kinetics as function of the plasma concentration of PAH with a "Km" of 53.2 mg/100 ml, and a "Vmax" (Tm_{PAH}) of 20.4 mg/kg/min. The total plasma protein concentration as observed before hydration was 4.28 ± 0.11 g/100 ml. The glomerular filtration rate, as measured by inulin clearance, was reduced in all experimental groups (Fig. 2) by 12 per cent in group 2 and by 28 per cent in group 3 indicating a dose-response relationship (Table 2). In group 3 a, the short-term exposure group GFR was reduced by 8 per cent.

Similar changes were observed in total plasma protein concentrations which were reduced in groups 2, 3 and 3 a by 14, 17 and 2 per cent, respectively. A dose-response relationship was indicated in groups 2 and 3 (Table 2).

The tubular excretion rate of PAH was not reduced at low plasma concentrations (<50 mg/100 ml) while it was reduced by 33 per cent at higher plasma concentrations (Fig. 2). The apparent Km was thus unchanged in this study but Tm_{PAH} representing the functional tubular transport capacity was considerably reduced. The renal concentrating capacity as measured by the urine osmolality after a maximal antidiuretic injection of lysin-vasopressin, was reduced in all experimental groups by approximately 15 per cent (Fig. 2). This corresponds well with the observed increased water consumption (Fig. 1).

Pathology

No macroscopic lesions were observed in the birds in groups 1, 2, 3 and 3 a.

Microscopic renal changes were constantly present in all birds receiving ochratoxin A, but the lesions were never severe. Furthermore the lesions did not differ significantly in the three experimental groups.

The histological picture of the kidneys was characterized by injury of the tubular epithelium accompanied by regeneration. The glomeruli in all the birds appeared morpho-

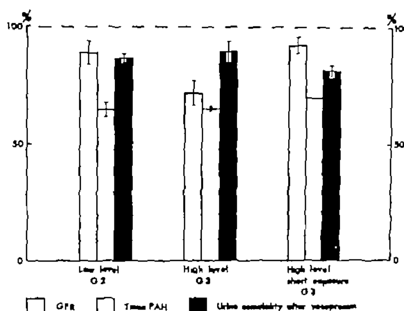


Fig 2 Changes in renal function.

TABLE 2. *Dose-Response Relation of Ochratoxin A*

Effect	n	a	b	r
Glomerular filtration rate (ml/kg/min)	17	3.06	−0.63	−0.43
Plasma protein (g/100 ml)	19	4.0[illegible]	0.52	−0.58
Residue of ochratoxin A (µg/kg) at slaughter				
Kidney	13	9.87	8.71	0.40
Liver	10	−0.20	5 5	0.60

Linear regression of various effects on feed concentration of ochratoxin A in the range 0.3–1 mg/kg (Linear regression equation $y = a + b \cdot x$; r = correlation coefficient)

logically normal. The lesions were mainly confined to the proximal and distal tubules of both reptilian and mammalian nephrons. The degeneration of the tubular epithelial cells was characterized by nuclear pyknosis and karyorrhexis and by separation of the tubular epithelium from the basement membrane which appeared normal (Fig 3). Also ballooning of epithelial cells and karyomegalic cells were frequently noted. The brush border appeared often slightly reduced compared with that in the controls (Fig 4). In virtually all the birds receiving ochratoxin A, mitotic figures, many of which were bizarre were seen in the tubules (Fig 3). In the proximal tubules, a number of strongly eosinophilic cells with very basophilic nuclei were observed. In cross sections of the tubules, these cells often appeared in pairs adjacent to one another. In the loops located in the medullary tracts, the distal tubules and the collecting tracts, only a few degenerated cells and mitotic figures were noted. Neither renal vascular lesions nor interstitial reactions were noted in any of the groups.

The livers were morphologically normal. In the majority of birds from all groups, including the control group, the hepatocytes displayed light inclusions in the cytoplasm. These inclusions represented accumulations of glycogen demonstrated by a PAS reaction combined with the diastase test.

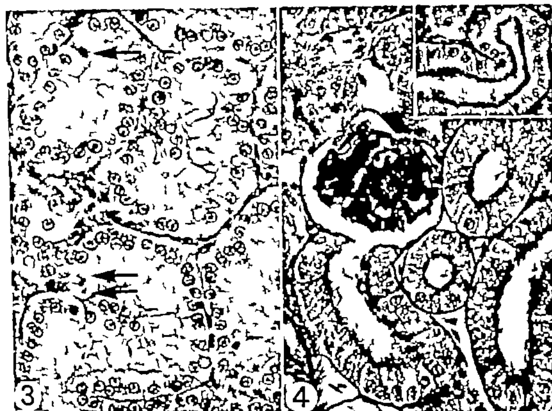


Fig. 3 Kidney from bird exposed to approximately 0.3 mg ochratoxin A per kg feed (group 2) during 341 days. Note the mitotic figure (arrow) and the pyknotic nucleus (double arrow) of the proximal tubular cells. The nuclei of the proximal tubular cells appear heterogeneously enlarged. H E, × 400.

Fig. 4 Kidney from bird of the same group as the bird in Fig. 3 Note the reduced brush borders in the proximal tubules. PAS, × 400. *Insert* Kidney from control bird with a well defined brush border of the proximal tubule PAS, × 250

Residues of Ochratoxin A

Ochratoxin A was not found in any of the tissues from the control birds. Ochratoxin A was found in increasing concentrations through the tissues Muscular liver and kidney (Fig. 5) A dose-response relationship of kidney and liver residues was observed in groups 2 and 3 (Table 2) Although groups 3 and 3 a were exposed to identical feed concentration of ochratoxin A, the residues were much higher in the short term group (Fig 5) similar to the findings in pigs exposed for a short time (*Krogh et al.* 1974)

The maximal concentration of ochratoxin A was 49.5 μg/kg in the kidney and 9 1 μg/kg in muscular tissue, both concentrations being found in group 3 a, the short term group.

Ochratoxin A was not found in any of the eggs analysed. Ochratoxin α, a metabolite of ochratoxin A, was not found in any tissue.

Causal Considerations

The feed content of oxalate was similar in all groups (Table 1) and citrinin and viridicatumtoxin was not detected at all thus, ochratoxin A appears to be the only observable nephrotoxic compound in the experimental feed. This is also supported by the dose-relationship of several effects on the feed concentration of ochratoxin A (Table 2)

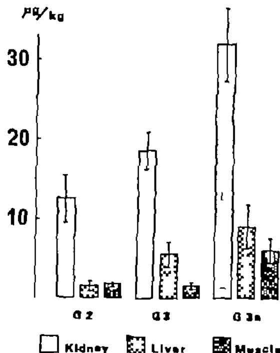


Fig 5 Residues of ochratoxin A in kidney liver and muscular tissue at slaughter

DISCUSSION

The present study confirms that ochratoxin A is a disease determinant of mycotoxic avian nephropathy as suggested by *Elling et al.* (1975) in a report on field cases of avian nephropathy

The observed changes of renal function are comparable with those encountered in ochratoxin A induced porcine nephropathy (*Krogh et al* 1974) In porcine nephropathy a renal loss of high-molecular weight proteins was observed a similar renal defect may be the explanation of the decreased total plasma protein concentration found in this study

The changes of renal structure were not as pronounced as those observed in experimental porcine nephropathy employing identical ochratoxin A exposure. More extensive changes of renal structure were found in the spontaneous cases of avian nephropathy where they were associated with feed borne ochratoxin A (*Elling et al.* 1975) This might indicate that additional nephrotoxic factors and/or higher levels of ochratoxin A were present in the feed and associated with the spontaneous development.

Renal manifestations of experimentally provoked ochratoxicosis in poultry have been observed previously Thus *Peckham et al.* (1971) observed development of visceral gout and nephrosis in an acute study employing ochratoxin A and B levels beyond those otherwise expected to occur naturally In a short term study involving feed levels of ochratoxin A in the range from 0.5 to 8 ppm, *Huff et al.* (1974) observed enlarged and pale kidneys, but the changes of renal structure were not further characterized. Renal impairment in chickens, indicated by measurements of phenol red clearance, was observed by *Huff et al* (1975) who used very high levels of ochratoxin A (4 and 8 mg per kg feed)

The tissue pattern of ochratoxin A residues was similar to that found in porcine nephropathy (*Krogh et al* 1974) the kidney containing higher concentrations than other tissues. The experimental birds in this study even birds containing residues of ochratoxin A up to 50 µg per kg, would have passed meat inspection because no macroscopical lesions were present. This aspect leads to considerations whether the meat inspection at poultry slaughterhouses will be able to prevent poultry contaminated with ochratoxin A residues from entering the human food channel. This represents a possible publi health problem.

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BRIEF REPORT

SEX DIFFERENCES IN RENAL DAMAGE INDUCED IN THE RAT BY THE FINNISH MUSHROOM *CORTINARIUS SPECIOSISSIMUS*

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Nieminen L. & Pyy K. Sex differences in renal damage induced in the rat by the Finnish mushroom, *Cortinarius speciosissimus*. Acta path. microbiol. scand. Sect. A, 84 222–224 1976.

The sensitivity of male and female rats to *Cortinarius speciosissimus* toxins was compared. Dried homogenized mushroom was given orally by stomach tubing at a dose of 250 mg dried mushroom/kg body weight. Both in males and in females, the kidneys were the only organs showing macroscopical changes. Sex differences in renal damage were observed in the area of the inner cortex.

Key words: Mycotoxins renal damage sex difference rat.

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In the last few years, several unusual cases of renal mushroom poisoning have occurred in Finland, the characteristic of which has been a latent period of several days before symptoms appear (*Hulmi et a.* 1974) It is generally suspected that *Cortinarius speciosissimus* has caused these poisonings. Identification of the mushroom responsibl for a given poisoning is often difficult, especially if the latent period is several days. However experiments in rats have substantiated th claims of the renal toxicity of *C speciosissimus* gathered in Finland (*Möttönen et al* 1975 *Nieminen et al.* 1975) Since in these previous experiments only male rats were used, in the present investigation we have compared the sensitiv ty of male and female rats to *C speciosissim*

Material and Methods

Seven mal and 16 female two-month-old Sprague-Dawley strain rats were used n the experiment The rats were fasted for 18 hours prior to the administration of the mushroom, but received water *ad libitum* Two mushrooms collected near Kuopio in the autumn of 1974 were dried with their stems and an aqueous suspension was prepared from this sampl (dry weight 1.8 g) using a Potter Elvehjem glass homogenizer 10 ml/kg of the suspension thus obtained (corresponding to 250 mg of dried mushroom/kg body weight) was given orally by stomach tube to the rats. In order to study the development of possible kidney damage in female rats, 3 rats were killed after one day 3 after 2 days, 3 after 3 days and the remaining 7 after 4 days. All the males were killed after 4 days, because the development of kidney damage induced by *C speciosissimus* male rats has been studied previously (*Nieminen et al.* 1975) The kidneys were removed nd weighed. The most seriously damaged kidneys were slightly enlarged. The ther organs were st died macroscopically and appeared normal Samples from the kidneys were fixed in neutral-formalin, embedded in paraffin, sliced into 8 µm sections and stained with haematoxylin-eosin.

Results

In the three females killed one day after application of the dose, no change were observed in the kidneys even microscopically

In all three females killed after two days, necrotic changes in the tubules throughout the inner cortex were observed Some of the tubular epithelial cells had hypereosinophilic cytoplasm and pycnotic nuclei *Epithelial cell* of this type were observed loose in the lumen of some of the tubuli.

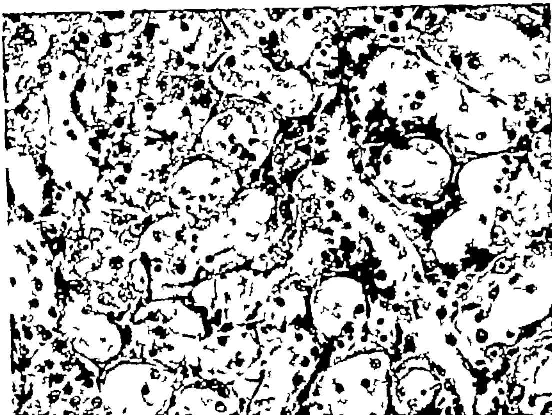


Fig. 1 Tubular necrobiosis and necrosis in the inner cortex. Collecting ducts are normal. Specimen from a female rat, 3 days post-administration. × 460

I[n] some of the epithelial cells the cytoplasm was vacuolated, the nucleus swollen, the nuclear membrane hyperchromatic or karyolytic. There were no changes in the tubuli of outer cortex. The size, shape and structure of the glomeruli were normal. In all three animals, some foci of cell lar interstitial infiltration were observed in the outer medullary zone near the cortical border. In addition to inflammatory cells and fibroblasts, fragments of disintegrated nuclei were observed. Early signs of necrosis were observed in the epithelial cells of the tubuli which were located in the foci of infiltration.

All three females killed three days after administration of the dose had damaged kidneys. I[n] two of these the changes were confined to the inner cortex where many of the tubular epithelial cells were necrotic. The third animal had severe damage both in the cortex and the medulla. I[n] the outer medullary zone large roundish foc[i] of inflammation were found in which, [in] addition to polymorphonuclear leucocytes mononuclear inflammatory [c]ells and fibroblasts were observed. In addition, scattered smaller interstitial infiltrates were observed peritubularly. The necrosis in the inner cortex was severe. In some tubules the cell bodies were disrupted so that only remnants remained in contact with the basement membrane. Signs of early necrosis were observed in the tubules of outer cortex. Some collecting ducts were dilated and contained eosinophilic cast.

One of the seven females killed after four days had no damage in the kidneys. Two had necrotic changes in many tubular epithelial cells in the cortex. Four had in addition to cortical necrosis inflammatory foci of [v]arious sizes or scattered interstitial infiltrates in the outer med[u]llary zone. In the inner cortical zone where the necrosis was most severe some mitotic figures indicative of regeneration were observed.

Three of the seven males killed after four days, had no damage in the kidneys. Three had scattered interstitial infiltrates in the outer medullary zone. N[o] changes could be observed in the cortex. In one signs of early necrosis were observed in the inner cortex. A few inflammatory foci of various sizes consisting of polymorphs, mononuclear inflammatory cells and fibroblasts in the outer medullary zone were also noted. Some collecting ducts were dilated.

Discussion

In the present study it was observed that *C speciosissimus* induced in male rats the same kind of renal damage as has been described earlier (*Nieminen et al.* 1975). In females, the interstitial infiltrates in the medulla were comparable with those seen in males. However the main lesions in females were the necrotic changes in the inner cortex. These were seen at the same time as the interstitial infiltrates in the medulla i.e. 2 days after application of the dose. Furthermore, in some females the only lesions seen were these necrotic changes in the inner cortex, no interstitial infiltration being found in the medulla. In the present and in earlier studies (*Möttönen et al.* 1975 *Nieminen et al.* 1975) necrotic changes were first seen in the kidney cortex of male rats 3–4 days after application of the dose, and were only seen in those animals which also had inflammatory foci in the medulla.

Jacobsen (1975) studied histochemically the activity of several enzymes in the proximal tubules of the kidneys in male and female rats. He observed sex differences in the activity of most enzymes in the descending part of the tubule, which is located in the inner cortex. I the outer cortex only a few enzymes showed sex differences. The sex differences found in the present study were also located in the inner cortex.

It is not known whether similar sex differences in the nephrotoxicity of *C speciosissimus* are found in man. Retrospective study of cases of accidental mushroom poisoning in man is unreliable, because of the difficulty in ascertaining the number of mushrooms consumed as well as the toxin content of the individual mushrooms. If it could be determined whether or not these sex differences are present in other species of experimental animals, it would be easier to speculate on the situation in man.

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INCREASE IN THE AMOUNT OF NUCLEAR RNA IN LIVER OF ASCITES TUMOUR-BEARING MICE

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Andersson, G. Christensson, E. & Heby O. Increase in the amount of nuclear RNA in liver of ascites tumour-bearing mice. Acta path. microbiol. scand. Sect. A, 84 225–234 1976.

The effect of tumour growth on the liver of the host was studied in the Ehrlich ascites tumour system. During the experimental period there was no infiltration of tumour cells in the liver and the increase in the proliferation rate of Kupffer cells and parenchymal cells was only small. An increasing stability of the liver cell nuclei towards disruption during the isolation procedure was found to accompany the ascites tumour growth. In parallel, there was an increase in the amount of nuclear RNA and in the cellular spermidine concentration. The major cause of these changes is probably an increasing demand on the liver by the growing tumour illustrated by the fact that an amount of protein exceeding that of the whole liver accumulates in the ascites fluid during tumour growth. A hypothesis according to which the cellular accumulation of spermidine may be involved in the stabilization of the cell nuclei and in the accumulation of nuclear RNA is advanced.

Key words: Nuclear RNA liver Ehrlich ascites tumour spermidine.

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In a previous study (*Andersson & Heby* 1972) we demonstrated that i.p. growth of an Ehrlich ascites tumour greatly influenced the spermidine concentration in the liver even though the liver did not become infiltrated with tumour cells. After an initial decrease the hepatic spermidine concentration increased during the remainder of the experimental growth period. We also noted that the hepatic DNA and RNA concentrations remained approximately constant during the entire growth period. In accordance with the latter *Hock Ligeti et al.* (1969) observed in a study of tumour free liver from cancer patients that the concentration of the nucleic acids did not change. They observed, however that the nuclear RNA content of the liver cells was significantly increased.

Since many observations indicate that spermidine is of importance for the stability of RNA, e.g. it can stabilize newly synthesized RNA towards degradation (*Raina & Jänne* 1968) it seemed likely that the striking changes in the hepatic spermidine concentration (*Andersson & Heby* 1972) would affect, if not the total RNA content, the nuclear RNA content of host liver. To study this possibility we have analysed the content of nuclear RNA in the livers of tumour-bearing mice as a function of time after the i.p. inoculation of Ehrlich ascites tumour cells.

Discussion

In the present study it was observed that *C. speciosissimus* induced in male rats the same kind of renal damage as has been described earlier (*Nieminen et al.* 1975). In females, the interstitial infiltrates in the medulla were comparable with those seen in males. However, the main lesions in females were the necrotic changes in the inner cortex. These were seen at the same time as the interstitial infiltrates in the medulla i.e. 2 days after application of the dose. Furthermore, in some females the only lesions seen were these necrotic changes in the inner cortex, no interstitial infiltration being found in the medulla. In the present and in earlier studies (*Möttönen et al.* 1975, *Nieminen et al.* 1975) necrotic changes were first seen in the kidney cortex of male rats 3–4 days after application of the dose, and were only seen in those animals which also had inflammatory foci in the medulla.

Jacobsen (1975) studied histochemically the activity of several enzymes in the proximal tubules of the kidneys in male and female rats. He observed sex differences in the activity of most enzymes in the descending part of the tubule, which is located in the inner cortex. In the outer cortex only a few enzymes showed sex differences. The sex differences found in the present study were also located in the inner cortex.

It is not known whether similar sex differences in the nephrotoxicity of *C. speciosissimus* are found in man. Retrospective study of cases of accidental mushroom poisoning in man is unreliable because of the difficulty in ascertaining the number of mushrooms consumed as well as the toxin content of the individual mushrooms. If it could be determined whether or not these sex differences are present in other species of experimental animals, it would be easier to speculate on the situation in man.

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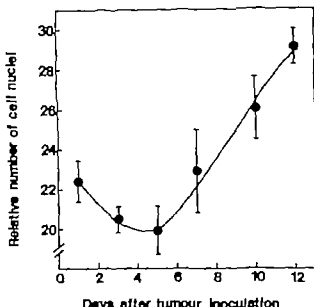


Fig 1 Relative number of cell nuclei (determined in a Bürker haemocytometer and defined as the mean of the number of nuclei per square) isolated from 1 g of mouse liver at various times after the i.p. inoculation of 4×10^6 Ehrlich ascites tumour cells. Means ± S.E.M. (n = 4)

With a view to autoradiography tumour-bearing mice received i.p. injections of 50 µCi of ^{3}H-thymidine 2 hours prior to killing. Small pieces of liver were fixed in buffered formalin and embedded in paraffin. 5 µ sections were covered with liquid photographic emulsion, Ilford K2 according to the dipping method. The film was exposed for 20 days, developed in Kodak D-19 for 5 minutes at 18° C, rinsed in tap water *for 10 seconds and fixed in* Kodak Unifix for 6 minutes at 18 C. After fixation, the sections were stained with Ehrlich acid haematoxylin and mounted in DePeX. The percentage of radio-labelled cells was estimated and only parenchymal cells and Kupffer cells were included in this *determination. Approximately 1500 cells,* randomly distributed, were counted in each section.

Determination of Protein in Cell-Free Ascites Fluid and in Host Liver

Ascites fluid was collected at different times of tumour development. The tumour cells were sedimented by centrifugation (2500 × *g* for 30 minutes) and the volume of the supernatant was measured. The total amount of protein in cell-free ascites fluid was determined according to *Lowry et al. (1951). The* protein content of host livers was determined on liver homogenates (1 part of tissue to 9 parts of 0.9 per cent NaCl) using the same method.

Chemicals

[5-^{3}H]-Uridine (specific activity 2.5 Ci/mmole) and ^{3}H-methyl-thymidine (specific activity 2.0 Ci/mmole) were purchased from The Radiochemical Center Amersham, England. Calf thymus DNA (type V) (Sigma Chemical Co St Louis, Mo., U.S.A.) and highly polymerized RNA (British Drug Houses, Pool, England) were used as standards in the nucleic acid analyses. Spermidine trihydrochloride was purchased from Calbiochem, Los Angeles, Ca., U.S.A., and Macaloid from Baroid Division, Tx, U.S.A. Autoradiographic nuclear *emulsion, Ilford K2, was obtained from Ilford,* England. Tris (tris-(hydroxymethyl)-aminomethane) and the other reagents used were all of analytical grade and were purchased from Merck, Darmstadt, West Germany

RESULTS AND DISCUSSION

Fig 1 shows the relative number of cell nuclei isolated from livers of tumour-bearing mice at various times after i.p. inoculation of 4×10^6 Ehrlich ascites tumour cells. The yield of cell nuclei reflects their stability towards disruption during the isolation procedure. Initially the yield decreased, but from day 5 through 12 it increased. Twelve days after tumour inoculation, the yield was 50 per cent greater than that obtained on day 5. Thus, as the tumour increased in size the stability of the liver cell nuclei of the host increased.

The amount of DNA in the cell nuclei obtained from 1 g of mouse liver obviously should be a function of the yield of

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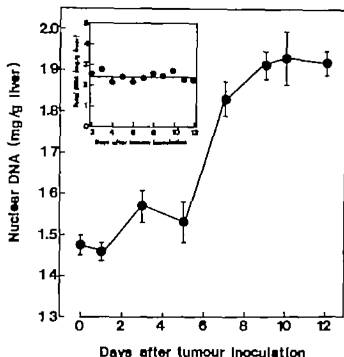


Fig 2 Amount of DNA in the cell nuclei isolated from 1 g of mouse liver at various times after the i.p. inoculation of 4 × 10^6 Ehrlich ascites tumour cells. Means ± S.E.M (n =) By way of comparison, the total amount of DNA per g of liver as determined in liver homogenates is shown (insert) Means ± S.E.M. (n = 4)

cell nuclei in the isolation procedure. Since the yield of liver cell nuclei changes during the progression of tumour growth, the amount of DNA contained in the cell nuclei isolated from 1 g of liver ought to change accordingly In fact this seemed to be the case inasmuch as a pattern of changes resembling that of the yield of cell nuclei was obtained (Fig 2) The amount of nuclear DNA per g normal mouse liver approximates the amount previously reported to apply to mice of the same strain and age (*Christensson & Lewan* 1974) Fig 2 also shows that the highest values, which occurred at the end of the experimental period, approach the values to be obtained if the total amount of DNA per g mouse liver is measured in liver homogenates (*Andersson & Heby* 1972 *Lewan* 1972, *Christensson & Lewan* 1974) This fact shows that there is a high yield of liver cell nuclei towards the end of the tumour growth period.

Fig 3 shows that the total amount of RNA (measured in liver homogenates) and the amount of cytoplasmic RNA (constituting the major portion of the hepatic RNA) remained relatively constant in the livers of tumour bearing mice during the entire tumour growth period The amount of RNA in the cell nuclei obtained from 1 g of mouse liver is obviously affected by the yield of cell nuclei in a way similar to that of nuclear DNA. This is seen in the amount of RNA extracted from the nuclei isolated by conventional 1 hour hydrolysis in 0.3 M KOH at 37 C (Fig 3) More RNA was extracted from the liver cell nuclei when they were subjected to an additional 15 hours of hydrolysis in 0 3 M KOH at 3 C and this fraction contained the greatest proportion of newly synthesized RNA, as shown in a study where the amount of ^{3}H uridine incorporated into nuclear RNA of livers of the tumour bearing mice was measured (Table 1) The "alkali-stable" fraction of RNA, which is very small in normal mouse liver increased progressively during tumour growth (Fig 4) The increase in the amount of alkali-stable nuclear RNA cannot be du to an increasing yield of liver cell nuclei nce there is an almost 25-fold increase i the amount of RNA as compared with the 1 5-fold increase in the yield of cell nuclei

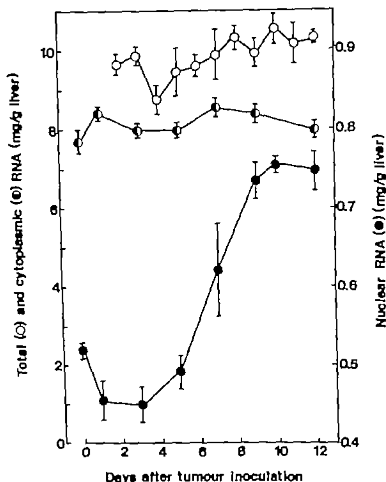


Fig. 3. Amount of RNA in the nuclear and in the cytoplasmic fraction, the two fractions obtained at the isolation of cell nuclei from 1 g of mouse liver at various times after the i.p. inoculation of 4×10^6 Ehrlich ascites tumour cells. Means ± S.E.M. (n = 3). By way of comparison, the total amount of RNA per g of liver as determined in liver homogenates is shown. Means ± S.E.M. (n = 4). The RNA was extracted by hydrolysis in 0.3 M KOH at 37 °C for 1 hour.

The alkali-stability of the RNA is probably illusive and may only reflect that the accessibility of RNA is of minor degree due to its occlusion in the citric acid-isolated cell nuclei. This idea is supported by the fact that citric acid induces chromatin condensation around the nucleolus and at the nuclear periphery (Fig. 5) (*Taylor et al.* 1973, *Christensson & Lewan* 1974) and that the additional amount of nuclear RNA in livers of tumour-bearing mice is only found if the cell nuclei are isolated by way of a medium containing citric acid. It is interesting to notice that *Hoch Ligeti et al.* (1969) had used a citric acid containing medium when they observed that the nuclear RNA content had increased in cancer patients.

It appears from our results that the yield of liver cell nuclei and thus the amount of DNA and RNA contained in the cell nuclei isolated from 1 g of liver increased during tumour growth. They also show that even though the changes in the total amount of nuclear RNA in livers of tumour-bearing mice conformed to the same pattern as changes in nuclear DNA, their relationship

TABLE 1 *Amount of ³H Uridine Incorporated into Nuclear RNA of Livers of Tumour Bearing Mice at Various Times after the i.p. Inoculation of 4 × 10⁶ Ehrlich Ascites Tumour Cells*

Time after tumour inoculation (days)	Incorporation of ³H-uridine		
	Fraction A* (CPM/μg RNA)	Fraction B* (CPM/μg RNA)	B/A
0	96.5 ± 4.8	n.d.	–
1	80.8 ± 8.9	n.d.	–
3	102.5 ± 17.9	n.d.	–
5	64.7 ± 13.3	76.3 ± 29.2	1.17
7	31.6 ± 4.7	41.2 ± 7.5	1.30
9	19.2 ± 2.6	30.0 ± 9.2	1.56
12	9.5 ± 0.3	17.8 ± 3.7	1.86

* RNA was extracted from liver cell nuclei in 2 consecutive hydrolysis steps. Fraction A was obtained by 1 hr of hydrolysis in 0.3 M KOH at 37 C and Fraction B by 15 hr of additional hydrolysis in 0.3 M KOH at 37 C. Each fraction was analysed for its radioactivity and RNA content. Means ± S.E.M. (n = 5)

n.d. = not determined. The amount of RNA extracted was too small to allow for an accurate determination of the CPM/μg RNA ratio.

changed during the progression of tumour growth, the increase in RNA being greater than the increase in DNA (Fig 6) Under the same experimental conditions we have previously noted a similar increase in the intracellular concentration of spermidine in livers of tumour-bearing mice (Fig. 7) Likewise an elevation of the hepatic spermidine concentration has been observed in rats harbouring an s.c. tumour (*Marton & Heby* 1974)

Spermidine as well as other naturally occurring polyamines such as putrescine and spermine, are known to stabilize nucleic acid structures and various membrane structures (for references see *Cohen* 1971 and *Bachrach* 1973) Therefore, many investigators have used media supplemented with polyamines for the isolation of cell nuclei (*Busch et al.* 1967 *Christensson & Lewan* 1974) Addition of putrescine spermidine or spermine to media used for the isolation and purification of nuclei and nucleoli from Walker 256 carcinosarcoma and liver tissue was found to preserve the nuclear and nucleolar structures, even with respect to ultrastructural morpho-

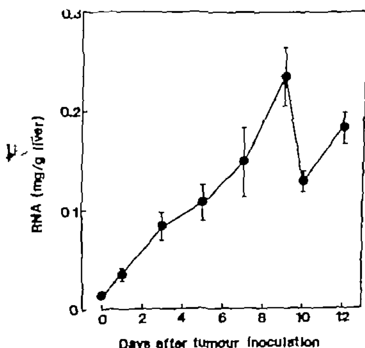


Fig 4 Amount of RNA (in the cell nuclei isolated from 1 g of mouse liver) not extractable by the standard 1 hr hydrolysis, but requiring a 16-hour hydrolysis in 0.3 M KOH at 37 C. Mean ± S.E.M. (n = 5)



Fig. 5 *Left* cell nuclei isolated in the absence of citric acid. The cell nuclei were isolated as described under *Materials and Methods* with the exception that medium A and B were replaced by 0.05 M Tris-HCl, pH 7.2, 10 mM KCl, 1 mM Na_2EDTA, 0.5 mM spermidine × 3 HCl, 2.8 mM KH_2PO_4, Macaloid (50 mg/l) In addition medium A contained 0.1 M sucrose and medium B 0.32 M sucrose. × 9 000 *Right* cell nuclei isolated in the presence of citric acid. The cell nuclei were isolated as described under *Materials and Methods* × 9,000

logy (*Busch et al* 1967) *MacGregor & Mahler* (1967) found that the polyamines were actually required for the preservation of nuclear morphology (i.e. to prevent lysis of the cell nuclei) in a cell-free system. Accordingly we observed an increasing yield of liver cell nuclei upon the addition of spermidine to the isolation medium (not shown) In view of these findings, it seems possible that the increasing stability of the liver cell nuclei during growth of the tumour is attributable at least partially to the increasing endogenous concentration of spermidine.

In addition to its possible stabilization of the liver cell nuclei, spermidine may play a role in the accumulation of nuclear RNA in the livers of tumour-bearing mice e.g. by stabilizing the RNA structure This is not without precedent, since spermidine if added to Ehrlich ascites cells, was found to stabilize newly synthesized RNA molecules towards enzymatic breakdown and to cause an accumulation of labelled RNA (*Raina & Jänne* 1968) If added to the perfusate spermidine was also found to produce an increase in the specific activity of nuclear RNA of perfused livers without changing the specific activity of cytoplasmic RNA (*Fausto* 1972) Thus, it

TABLE 2. *Percentage of Cells Labelled by ³H Thymidine in Mouse Liver at Various Times after the i.p Inoculation of 4 × 10⁶ Ehrlich Ascites Tumour Cells*

Time after tumour inoculation (days)	Labelling index (%)			
	Parenchymal cells		Kupffer cells	
	(Range)	Mean§	(Range)	Mean§
0	(0 1 0.3)	0..	(0.3–0 4)	0 4
2	(0 3 0 9)	0 6	(0.8–2.4)	1.6
6	(0 4 0.8)	0 6	(2.8–3.0)	2.9
12	(0.8 1 2)	1 1	(1.2 2.5)	1 7

Number of parenchymal or Kupffer cells labelled with ³H thymidine in relation to the number of unlabelled counterparts expressed in per cent.
§ Mean of 2 3 mice

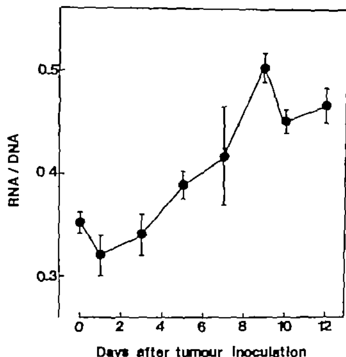


Fig 6 Nucleic acid ratio (mg RNA/mg DNA) in liver cell nuclei isolated from tumour-bearing mice at various times after the i.p. inoculation of 4 × 10⁶ Ehrlich ascites tumour cells. Means ± S.E.M. (n = 5)

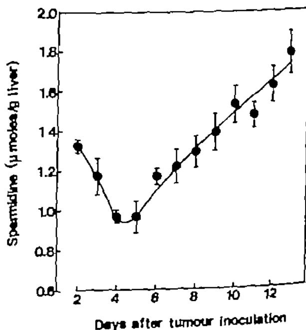
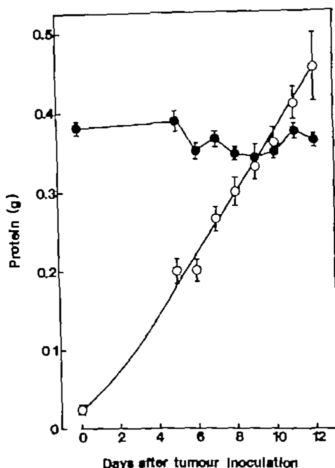


Fig 7 Spermidine concentration in liver of tumour-bearing mice at various times after the i.p. inoculation of 4 × 10⁶ Ehrlich ascites tumour cells. Means ± S.E.M. (n = 4)

seems possible that spermidine is involved in the observed accumulation of nuclear RNA.

The changes observed in the livers of tumour-bearing mice may reflect both an immunological response of the reticulo-endothelial system, i.e. the Kupffer cells, to tumour antigens, and a response of the parenchymal cells to the tumour burden. In the present study the liver weight remained relatively constant during the entire growth

Fig 8 Total amount of protein in cell-free ascites fluid (○—○) and in liver (●—●) of tumour-bearing mice at various times after the i.p. inoculation of 4 × 10^6 Ehrlich ascites tumour cells. Means ± S.E.M. (n = 5)



period (not shown) The percentage of Kupffer cells labelled with ^{3}H-thymidine was 3 per cent at the most during i.p tumour growth (Table 2) being 14 per cent during growth in the lungs (*Baserga* & *Kisieleski* 1961) Thus, the immunological response was considerably lower in tumours growing i.p. than in tumours growing in the lungs. The percentage of parenchymal cells labelled with ^{3}H thymidine was maximally 1 1 per cent (Table 2) and, in the same range as the percentage obtained when the tumour grew in the lungs (*Baserga* & *Kisieleski* 1961)

Baserga & *Kisieleski* (1961) observed that, in accordance with the higher proliferating activity of the Kupffer cells in relation to the parenchymal cells, the ratio of parenchymal cells Kupffer cells, which was 0.8 in control mice, decreased rapidly after the i.v injection of the Ehrlich ascites tumour cells from the 7th day and onwards, the ratio was approximately 0.5 In the present study the changes in the ratio of parenchymal cells Kupffer cells were considerably less pronounced. This observation is in accordance with the fact that the proliferating activity of the Kupffer cells was much lower in tumours growing i.p. than in tumours growing in the lungs. The ratio of parenchymal cells Kupffer cells was 0.65 in control mice and decreased only slightly after the i.p. injection of the Ehrlich ascites tumour cells. From day 6 through 12, the ratio was approximately 0.5

The moderate increase in the rate of proliferation of parenchymal cells and Kupffer cells cannot account for the observed increase in the nuclear RNA content. To explain the increase in the nuclear RNA content, the

additional phenomena to accompany the ascites tumour growth have to be considered, for instance, the increased synthesis of such plasma proteins as accumulate in the ascitic fluid (Fig 8) In general, the increased demand on the liver by the growing tumour is probably the major cause of the increase in nuclear stability and in the accumulation of nuclear RNA. Because of the particular properties of spermidine it seems likely that the cellular accumulation of spermidine may be involved in the stabilization of the cell nuclei and in the accumulation of nuclear RNA.

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THE ROLE OF THYMUS FOR THE DEVELOPMENT AND PROGNOSIS OF HYPERTENSION AND HYPERTENSIVE VASCULAR DISEASE IN MICE FOLLOWING RENAL INFARCTION

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Svendsen, U. G. The role of thymus for the development and prognosis of hypertension and hypertensive vascular disease in mice following renal infarction. Acta path. microbiol. scand. Sect. A, 84 235–243 1976

Partial infarction of one kidney and contralateral nephrectomy was followed by a rapid and significant increase in blood pressure both in haired mice with a normal thymus function and in nude mice with genetical aplasia of the thymus. The level of blood pressure and the prognosis were not influenced by the presence of thymus within the first 3 months after partial infarction of the kidney, but a significantly more pronounced increase in blood pressure after 4 months was observed in a small group of haired mice than in a similarly treated small group of nude mice. A marked degree of round cell infiltration around intrarenal arteries was only found in the haired mice commencing 2 months after partial infarction of the kidney. Degenerative changes were observed in the kidneys of the haired mice 2–4 months after infarction. Very few of these lesions were found in the nude mice. Attempts to transfer the hypertension by means of viable lymph node cells from hypertensive donors to normotensive syngeneic recipients failed. The results support the assumption that high intravascular pressure induces thymus-dependent immune reactions against substances in the vascular walls which, in turn, may have a prognostic significance. The results give no support for the assumption that the earlier phase of the hypertension which follows partial infarction of the kidney and contralateral nephrectomy is thymus-dependent.

Key words: Hypertension; thymus; renal infarction.

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Evidence that thymus dependent immune reactions have pathogenic importance for the round cell infiltration around hypertensively damaged arteries, has come from experiments in which it was possible to transfer a changed cellular reactivity against damaged arteries by means of washed thoracic duct cells from either acute angiotensin-II hypertensive donor rats (9) or chronic Goldblatt 2 kidney hypertensive donor rats (15) to normotensive syngeneic recipients. Further support for this as-

sumption has come from experiments in mice with either DOCA and salt hypertension (14) or Goldblatt 1 kidney hypertension (15) in which a marked difference in the degree of round cells around damaged arteries in the heart or the kidney was found between athymic mice and their normal littermates. *Okuda & Grollman* (8) (1967) reported that the level of hypertension in "Loomis hypertensive" rats (one kidney partly infarcted and contralateral nephrectomy) was less pronounced following thymectomy compared with the level in non thymectomized Loomis hypertensive rats. They further found that it was possible to transfer the hypertension by means of viable lymph node cells from these hypertensive rats into normotensive recipient rats, which were rendered immunologically tolerant by neonatal pre treatment with donor rat spleen cells. It was proposed that the result gave added support both to the concept of an immunological mechanism as the basis of this form of hypertension and to the suggestion that a similar mechanism may account for some forms of human hypertension which accompany destructive lesions of the kidney (8)

The aim of the present study was to compare the degree and prognosis of the hypertension as well as the hypertensive vascular disease in Loomis hypertensive athymic mice and similarly hypertensive normal mice. Mice would seem suitable for this investigation because of the existence of a mutant nude strain, which lack recognizable thymus tissue ... have no or nearly no (10) thymus-derived lymphocytes, as estimated both by functional and histological criteria (for literature see 11)

Furthermore it was investigated whether or not it could be confirmed that Loomis hypertension can be transferred by viable lymph node cells. For this purpose, lymph node cells were transferred from either Loomis hypertensive or normotensive donor mice to syngeneic normotensive recipient mice.

MATERIAL AND METHODS

The investigation was performed in two parts.

Part I Studies Elucidating the Importance of Thymus for the Degree and Prognosis of Hypertension and Hypertensive Vascular Disease

Animals outbred NMRI mice (SPF Gl. Bomholtgaard Ltd., Denmark) of both sexes, 20–25 g, carrying the mutant allele (nu/nu) and their haired littermates (nu/+) received tetracyclin in the drinking water (100 mg/l)

The animals were divided into groups.

Group 1 20 nude and 20 haired untouched control mice 10 males and 10 females in each group

Group 2 36 haired and 31 nude male mice with the left kidney partly infarcted, placed subcutaneously and contralateral nephrectomy (6) The mice in group 2 were placed at random in cages, and the mice in a cage were investigated after 5 15 30, 60 90 and 1 0 days. The further treatment was the same for both groups. Before sacrifice blood pressure was recorded. In light ether anaesthesia, a catheter was placed in the left carotid artery and connected to a Tybjærg-Hansen capacitance pressure transducer (Simonsen & Weel, Copenhagen) and a G-14 graphic recorder (Danbridge, Copenhagen) Blood pressure was recorded for one hour in the conscious semirestrained animal while it was placed in plastic tube.

After sacrifice the relative heart weight (heart weight × 100/body weight) and, as regards the mice in group 2, the size of the kidney infarct in percentage of the whole kidney was determined. The heart, kidney and pancreas were fixed in 4 per cent formalin and embedded in paraffin. Five micron thick sections were cut and stained with the van Gieson Hansen (VGH) and the periodic acid Schiff (PAS) stain The round cell infiltrations around arteries in the heart and the arcuate/interlobular arteries in the kidney were graded semiquantitatively according to photographed scales from 1+ to 3+ in which a 1+ cellular reaction includes the sparse mononuclear reaction in ad ventitia of normal arteries (14 15)

Part II Studies Elucidating the Ability to Transfer the Hypertension by Lymph Node Cells

Animals SPF inbred Balb c/A and C_3H/Tif male mice, 20–25 g from Gl Bomholtgaard Ltd Denmark, were fed mouse pellets and received tap water *ad libitum*

Skin transplantation was performed to measure the degree of histocompatibility by grafting squares of approximately 10 × 10 mm skin from donors to the dorsal thoracic wall of recipients. Grafted

animals were housed singly in boxes and were routinely observed several times a day for the appearance of the graft. The grafts were registered as rejected when they appeared as scabs. 10 Balb/c/A mice (H-2d) were challenged with one allograft (C3H/Tif, H-2K) and one isograft.

Donor Balb/c/A mice were made "Loomis" hypertensive. Other mice, selected at random, served as control donors. Eight weeks later *direct blood pressure* was recorded and the further treatment was as described above.

The cervical, inguinal, axillary and mesenterial lymph nodes from the donors were gently homogenized in a Potter Elvehjem, filtrated and washed twice in sterile *Minimum essential medium eagle* (Flow Laboratories, Scotland) resuspended in 1 ml of medium and injected intraperitoneally into the recipients. Estimations of the number of living and dead lymphocytes in the cell suspensions were performed by way of supravital dye exclusion (1, 5). Each recipient received 25–50 × 10⁶ viable lymph node cells intraperitoneally from one donor. Each donor gave cell suspensions to one or two recipients. One to two days before the cell transfer the recipients were unilaterally nephrectomized. The recipients were divided at random into two groups. One group received lymph node cells from hypertensive donors and one group from normotensive donors. After the cell transfer the recipients lived for 15 and 40 days before direct blood pressure was recorded. After sacrifice, the relative heart weight was determined and histological investigations performed as described above. For comparison of experimental results, the students t-test was used. The five per cent level was used as indicative for significance of differences.

RESULTS

Part I Studies Elucidating the Importance of Thymus for the Degree and Prognosis of Hypertension and Hypertensive Vascular Disease

No thymus was found in any of the nude mice after dissection of the mediastinal region post mortem, whereas the thymus was present in all haired mice.

Group 1, control mice: the mean blood pressure in mice of both sexes was the same ($p > 0.5$) in the nude (114 ± 14 mmHg (SD) (range 90–140 mmHg)) and the haired mice (113 ± 13 mmHg (range 90–140 mmHg)). *The mean relative heart weight* in the nude male and female mice was 0.43 ± 0.05 per cent (range 0.35–0.54), significantly greater ($p < 0.005$) than in the haired mice (0.35 ± 0.03 per cent (range 0.33–0.43)).

Microscopic investigation: the heart, kidney and pancreas: no vascular disease.

Group 2: Loomis hypertensive nude and haired mice. Only mice surviving the first 48 hours after the operation are included in the material. The mortality within the first 48 hours was the same in nude and haired mice (35–40 per cent). Later only few mice died (around 20 per cent, equal in nude and haired mice). Fig. 1 a and b show the *mean blood pressures* in the nude and haired mice compared with the mean blood pressure obtained in the control groups of mice. It is seen to apply to both groups that the operation is followed by a significant increase in blood pressure, which also varied similarly in the nude and haired mice ($p > 0.05$) until day 90. However, at day 120 a significant difference was found, the haired mice showing a more marked secondary increase in blood pressure than the nude ones. *The mean relative heart weight* was significantly increased both in nude (0.50 ± 0.04 per cent (range 0.45–0.53), $p < 0.025$) and haired (0.52 ± 0.04 per cent (range 0.45–0.56), $p < 0.001$) mice already 5 days after the operation. The relative amount of infarcted kidney decreased from around 45 per cent (day 5) to around 20 per cent (day 120).

Microscopic investigation: the heart: fibrinoid necrosis (PAS positive deposits in the entire thickness of the vessel wall with loss of normal structures) of the whole circumference in the small arteries was observed both in nude and haired mice 5 days after infarction. Later only few nude and haired mice showed increased PAS positivity of the vessel walls. *Infarcts* of the heart muscle were found in 8 of 35 (23 per cent) haired and 6 of 31 (20 per cent) nude mice. Increased numbers of *round cells* of a morphology like that of lymphocytes or monocytes, around damaged arteries were found in 5 haired mice (2 with 3+ and 3 with 2+ cellular reactions) but not in any of the nude mice. Increased amounts of *connective tissue fibrils* concentric around the arteries were observed both in

nude and haired mice in which hypertension had been manifest for more than 30 days.

TABLE 1 *Round Cell Infiltration Around Intrarenal Arteries*

Day	A. interlobularis/arcuata nude mice	A. interlobularis/arcuata haired mice
	+	+ +
	+	+
5	+	+
	+	+
	+	+
	+	+ +
	+	+
15	+	+ +
	+	+
	+	+
	+	+
	+	+
	+	+
30	+	+
	+	+
		+
		+
	+	+ +
	+	+ + +
	+	+ + +
	+	+ + +
60	+	+ +
	+	+
	+	+ + +
		+ + +
		+
	+	+ +
	+	+
90	+	+ +
	+	+ +
	+	+
	+	+ + +
	+	+ + +
120	+	+ + +
	+	+ + +
		+ + +

The degree of round cell infiltration around the interlobular/arcuate arteries in the kidney of Loomis hypertensive nude and haired mice. The day indicates at which time after partial infarction the animals are sacrificed. A marked degree of round cells are found in haired mice commencing 60 days after partial infarction.

The kidney fibrinoid necroses were observed in a few haired mice in the arcuate/interlobular arteries. Table 1 shows the number of *round cells* observed around these arteries. No nude mice, but 18 out of 36 haired mice showed increased numbers of round cells of a morphology like that of lymphocytes or monocytes (10 with a 3+ and 8 with a 2+ cellular reaction) (Fig 2 A and B). The round cell infiltration in the haired mice was most pronounced after day 60. Increased amounts of *connective tissue fibrils* concentric around the arteries were found both in nude and haired mice which had had hypertension for more than 30 days. After 60 days, thickened and wrinkled basement membranes of the tubules, dilated tubules, hyaline casts, glomerular lesions consisting of partly hyalinized tufts, and interstitial fibroses were observed frequently in the haired mice (Fig 2 C). Very few of these lesions were observed in the nude mice (Fig 2 D). *The pancreas*: no vascular disease.

Part II Studies Elucidating the Ability to Transfer the Hypertension via Lymph Node Cells

Skin transplantation All mice accepted the isografts and hair had begun to grow at the termination of the experiment. All allografts were rejected after a medium time of 14 days.

In the *groups of donor mice* a significant difference between the mean blood pressure and the mean relative heart weight was observed in the group of Loomis hypertensive and normotensive ($p<0.001$) mice (Table 2 A and B). The kidney infarct varied between 20–50 per cent of the kidney. *The histological investigation* showed vascular disease in the heart and kidney of the Loomis hypertensive mice as described above whereas no vascular disease was observed in any of the normotensive donor mice. Table 2 A and B further show that 15 and 40 days after the cell transfer no significant difference in the mean blood pressure ($p>0.1$) in groups of recipient mice which had received lymphocytes from Loomis hypertensive and in those

TABLE 2. *Effect of Lymph Node Cell-Transfer on Mean Blood Pressure and Mean Relative Heart Weight*

A. Mean blood pressure

Donors		Recipients	
Type	mean BT (mmHg ± SD)	mean BT (mmHg ± SD) 15 days	40 days
Loomis hypertensive mice	152 ± 8 (19) (*range 140–170*)	135 ± 10 (10) (*range 120–155*)	137 ± 11 (15) (*range 120–160*)
Normotensive mice	1 7 ± 7 (15) (range 115–140)	135 ± 8 (10) (range 120–145)	132 ± 7 (11) (range 125–140)

B. Mean relative heart weight

Donors		Recipients	
Type	mean relative heart weight (per cent ± SD)	mean relative heart weight (per cent ± SD) 15 days	40 days
Loomis hypertensive mice	0.48 ± 0 04 (19) (range 0.42–0.56)	0 44 ± 0.03 (10) (range 0 40–0.48)	0.43 ± 0.02 (15) (range 0 40–0 47)
Normotensive mice	*0.43 ± 0.04 (15)* (range 0.32–0 47)	*0.42 ± 0 02 (10)* (range 0.40–0 44)	*0.44 ± 0.03 (11)* (range 0.40–0.50)

Lymph node cells (25–30 × 10⁶) from Loomis hypertensive donor mice were given intraperitoneally to normotensive recipient mice. Blood pressure and mean relative heart weight were measured 15 and 40 days after treatment. SD standard deviation. Number in brackets number of mice.

who received cells from normotensive donor mice could be observed Similarly no difference in the mean relative heart weights was found ($p > 0.1$)

Histological investigations of the heart, kidney and pancreas of the recipients revealed no vascular disease, and especially no increased cellular reactions around the vessels.

DISCUSSION

Partial infarction of one kidney and contralateral nephrectomy were rapidly succeeded by a significant and similar rise in blood pressure both in athymic nude and normal haired mice. The course of the blood pressure curve (Fig 1 A and B) in the first 10 weeks was the same as that found by others in mice using the tail plethysmograph method for measuring the blood pressure (2) The result shows that this form of hypertension is independent of the presence of thymus and thymus-derived lymphocytes, at least in the first 90 days, as previously found to apply to Goldblatt one-kidney hypertension in mice (15) The induced hypertension was of benign type (few heart infarcts and spontaneous deaths) both in nude and haired mice, contrary to the malignant one-kidney Goldblatt hypertension previously induced (15) The relative size of the kidney infarct was the same in nude and haired mice. Although the mean blood pressure varied similarly in nude and haired mice, round cell infiltrations around the interlobular and the arcuate arteries of the uninfarcted part of the kidney was found only in the haired mice, commencing after 60 days (Table 1) One hundred and twenty days after partial infarction of the kidney a marked secondary hypertension and pronounced vascular disease in the kidney was found in the remaining

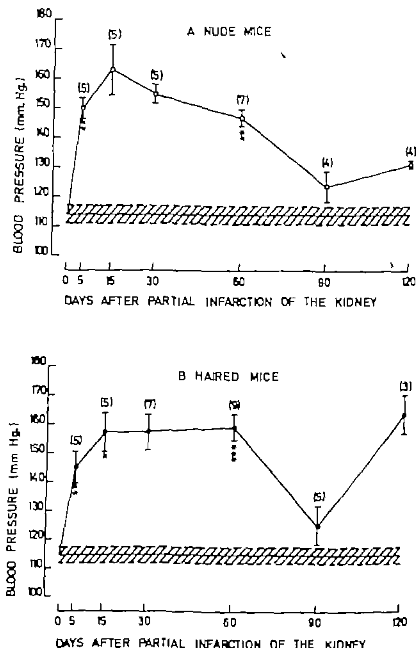
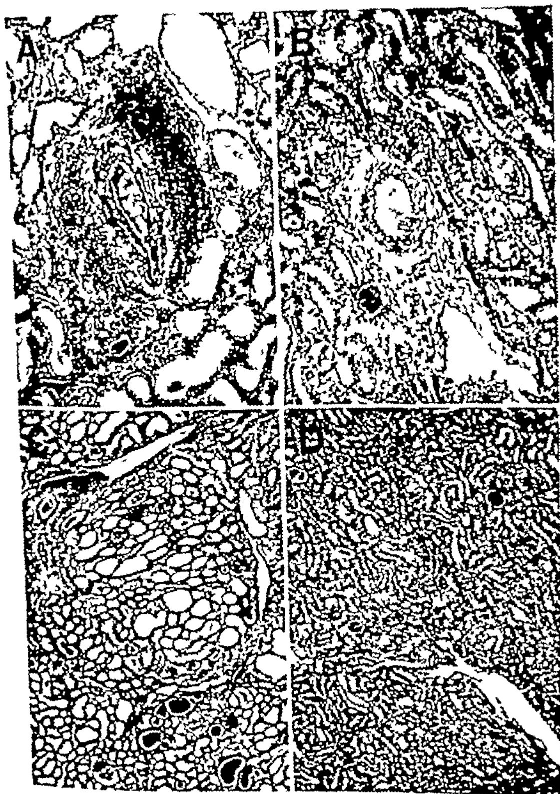


Fig 1 The mean blood pressure ± SEM (standard error of the mean, student's t test) in nude (A) and haired (B) mice with partial infarction of one kidney and contralateral nephrectomy The scratched area indicates the mean blood pressure in the nude (A) and the haired (B) control mic ± SEM. After 120 days, a significant ($p<0.001$) difference between the mean blood pressure of the nude and the haired mice was observed. Number in brackets number of mice. *** $p<0.001$ * $p<0.05$ (student's t-test)

Fig 2 An arcuate artery in the kidney of a haired mouse (A) and a nude mouse (B) 120 days after partial infarction of the kidney and contralateral nephrectomy In (A) a 3+ round cell infiltration, fibrinoid degeneration of the vessel wall and media hypertrophy with increased ratio wall/lumen is apparent. Degenerative changes, consisting of glomerular changes and dilated and degenerated tubules with hyaline casts are seen in the haired mouse kidney (C) In the kidney from the nude mouse (D) no changes are seen. PAS staining 140 × (A and B) 56 × (C and D)


A
B
D

3 haired mice, contrary to the remaining 4 nude mice which had a significantly lower blood pressure and no vascular disease in the kidney Two out of 5 haired mice died spontaneously at this time, presenting marked degenerative changes in the kidneys. These degenerative changes in the kidneys were observed only in the haired mice in which hypertension had persisted for more than 60 days, being most marked after 120 days. At this time they could be responsible for the observed difference in blood pressure in nude and haired mice in a way similar to the effect of vascular disease in the untouched kidney of Goldblatt two-kidney hypertensive rats (3 17) The changes in the kidneys of the mice observed in the present study were morphologically similar to ischaemic changes (7) and to those found to occur in DOCA/salt hypertensive mice (14) and rats (12) in the untouched kidney in two-kidney hypertensive rats (16) and in the uninfarcted part of the kidney in Loomis hypertensive rats (6) Fibrinoid degeneration and the necroses of the arteries in the heart were only found in the mice investigated 5 days after infarction of the kidney possibly due to the rapidly increased blood pressure (for literature see *Giese* 1966) The finding of a similar increase in blood pressure following partial infarction of the kidney in athymic and normal mice disagrees with the findings obtained by *Okuda* & *Grollman* (8) who observed a significantly lower blood pressure in adult thymectomized Loomis hypertensive rats than in Loomis hypertensive rats with untouched thymus.

Transfer of viable lymph node cells from Loomis hypertensive donors to normotensive syngeneic recipients was not followed by any increase either in the mean blood pressure or in the mean relative heart weight of the recipients. Nor were any round cell infiltrations found in the organs from the recipients. This failure of transfer of the hypertension is in contrast to the successful transfer in rats found by *Okuda* & *Grollman* (8) This discrepancy could be due to a species difference, but the interpretation is difficult, since *Okuda* & *Grollman* did not use inbred rats, nor did they control the degree of the tolerance which they believe to have induced, and finally a histological description of the organs in the donor and recipient rats was not reported.

The present investigation supports the evidence obtained by way of acute angiotensin II hypertension (9 13) chronic DOCA/salt hypertension (14) and Goldblatt one (15) or two- (13) kidney hypertension, namely that high intravascular pressure results in thymus-dependent immune reactions against substances in the vessel wall which become antigenic when high intravascular pressure occurs. The possibility exists that the immune reactions during prolonged hypertension, as a secondary event, could induce progressive ischaemia of the kidney due to the constrictions of intrarenal arteries, cf. oedema of the media with decreased ratio wall/lumen (see Fig 2 A) In this way the immune reactions may have a pathogenic importance for the development of hypertensive nephropathia, and for a secondary increase in blood pressure of prognostic significance

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THE MYOCARDIAL CAPILLARY VASCULATURE IN EXERCISING ANIMALS WITH INCREASED CARDIAC PRESSURE LOAD

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Ljungqvist, A., Unge G & Carlsson, S. The myocardial capillary vasculature in exercising animals with increased cardiac pressure load. Acta path. microbiol scand. Sect A 84 244–246, 1976.

The myocardial capillary reaction was studied in normal rats subjected to swimming exercise and in rats in which aortic stenosis had been produced at various time intervals before the swimming exercise was commenced. The data obtained indicated that neoformation of myocardial capillaries during swimming exercise occurred only in rats without aortic stenosis. It is concluded that a heart subjected to an increased pressure load is less able to respond to a superimposed volume load by an increase in its capillary supply than is a normal heart.

Key words Myocardial capillaries exercise aortic stenosis.

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In earlier studies of various forms of cardiac hypertrophy different reactions were observed in the myocardial capillary vasculature. Thus, a neoformation of myocardial capillaries was demonstrated in cardiac hypertrophy secondary to exercise (*Petrén et al.* 1936, *Tittle et al* 1966 *Leon & Bloor* 1968, *Tomanek* 1970 *Ljungqvist & Unge* 1972 and 1973) whereas no such vascular reaction was observed in cardiac hypertrophy induced by aortic stenosis and arterial hypertension (*Ljungqvist & Unge* 1972 and 1973) These findings raise the question whether swimming exercise will give rise to a capillary neoformation in hearts which are already under increased pressure load from aortic stenosis or hypertension

MATERIAL AND METHODS

Twenty-nine female Sprague-Dawley rats were used in these experiments Five of the rats were excluded however due to technical failures. The rats were housed in cages with 5 rats in each and were given laboratory diet and water *ad libitum*.

Cardiac hypertrophy was induced by the application of a 0.5 mm wide silver clip on the descending aorta immediately below the diaphragm.

The animals were divided into four groups.

I 8 rats were killed 6 weeks after the operation. During the last 4 weeks the rats had been subjected to swimming exercise for one hour each day 6 days per week as previously described (*Ljungqvist & Unge* 1972)

II Six rats were killed 12 weeks after the operation and after they had been subjected to swimming exercise during the last 4 weeks.

III Six non-operated normal rats were exercised for 4 weeks.

IV. Six non-operated and non-exercised normal rats served as controls of groups I–III.

All rats were of identical age at the time of killing. On the last day of the experiment, the body weight of each rat was determined and 2 μCi ^{3}H-thymidine/g body weight was injected intraperitoneally (specific activity 5.0 Ci/mM, Amersham, England). The rats were anaesthetized by intraperitoneal injection of Nembutal. The aorta was exposed and a catheter inserted and directed towards the heart. Fixation of the heart was performed by perfusion via the catheter using 1.5 per cent glutaraldehyde solution buffered by Na-cacodylate to pH 7.4. During this procedure the animals died. The hearts were removed, the atria dissected away and the ventricular portion of the hearts weighed for determination of the heart/body weight ratios. In Table 1 these ratios are multiplied by 1000 for practical reasons.

After post-fixation in 4 per cent glutaraldehyde solution, the specimens were embedded in paraffin and 4 μ thick transverse sections were taken and placed on glass slides. The sections were covered with Kodak AR 10 film for autoradiography according to the stripping film technique. The films were exposed for 28 days at +4 °C, developed in Kodak A 19B and fixed in Kodak acid fixer. The sections were then stained through the film with haematoxylin-eosin.

The autoradiograms were examined under a high power oil immersion lens in an ordinary binocular light microscope in which one eye piece was equipped with a square field. ^{3}H thymidine labelled capillary wall cells from 200 fields were counted. The procedure was repeated once, and the mean of the two countings calculated. Classification of capillary wall cells was done according to the same criteria as those described by *Ljungqvist & Unge* (1973). The material was coded in order that the examiner might remain ignorant of the history in each case.

TABLE 1. *Heart/Body Weight Ratios (× 1000) in Rats with Aortic Stenosis Persisting for 6 Weeks and Subjected to Swimming Exercise during the Last 4 Weeks (Group I), in Rats with Aortic Stenosis Throughout 12 Weeks Including 4 Terminal Weeks when they were Subjected to Exercise (Group II) and in Rats Subjected to Exercise for 4 Weeks only (Group III). Group IV is Composed of Normal Controls*

Group	No. of rat	Ratio
I	6	3.97 ± 0.34
II	6	3.86 ± 0.25
III	6	4.30 ± 0.32‡
IV	6	3.79 ± 0.26

The figures are means ± SE.
* Significantly different from controls (0.05 > p > 0.01)
‡ Significantly different from control (0.01 > p > 0.001)

TABLE 2. *Labelling Indices of Myocardial Capillary Wall Cells after ^{3}H Thymidine Injections into Rats with Aortic Stenosis Throughout 6 Weeks and Subjected to Swimming Exercise during the Last 4 Weeks (Group I), in Rats with Aortic Stenosis Throughout 12 Weeks Including 4 Terminal Weeks when they were Subjected to Exercise (Group II) and in Rats Subjected to 4 Weeks of Exercise only (Group III). Group IV is Composed of Normal Controls*

Group	No. of rats	Index
I	6	49.0 ± 10.3
II	6	51.0 ± 10.4
III	6	84.0 ± 17.8*
IV	6	44.0 ± 18.3

The figures are means ± SE.
* Significantly different from control ($p < 0.001$)

RESULTS

The heart/body weight ratios of the various groups of rats are shown in Table 1. It appears that there is a significant increase of the ratios (= cardiac hypertrophy) in the experimental groups II and III as compared with rats in the control group IV, but not in the experimental rats in group I. Furthermore, the degrees of cardiac hypertrophy was significantly higher in group III than in group II.

In Table 2, the labelling indices of myocardial capillary wall cells in the various groups of rats are listed. As compared with the control rats in group IV, the increase in labelling index is highly significant in rats in group III, thus indicating a capillary neoformation in these hearts. As regards the rats in groups I and II, however, no increase in labelling index was recorded.

DISCUSSION

The results of the present study have confirmed our previous observations of the fact that swimming exercise leads to a significant degree of cardiac hypertrophy and myocardial capillary neoformation in rats (*Ljungqvist et al.* 1973) In our previous studies, capillary neoformation was found to be at a low level or non-existing in cases of cardiac hypertrophy induced by aortic stenosis (*Ljungqvist et al* 1973)

In the presence of aortic stenosis, the heart is subjected to an increase in pressure load, during swimming exercise being subjected to an increase in volume load. In both conditions, the myocardial bulk will increase to meet the increased functional demands. To judge from the present observations, a relatively moderate degree of cardiac hypertrophy induced by pressure load will not be further influenced by a superimposed increase in volume load, the ultimate degree of cardiac hypertrophy being significantly less than that induced by an increased volume load alone. This suggests that the type of cardiac hypertrophy induced by an increase in pressure load is basically different from that resulting from an increase in volume load and once the first type of hypertrophy has developed, the myocardium is less capable of responding to stimuli of the latter type. A further feature of this situation was the absence of any capillary neoformation during swimming exercise to which rats with a previous history of aortic stenosis were subjected.

In the present series of experimental animals no spontaneous deaths occurred. Nor were any obvious signs of myocardial insufficiency in the experimental animals recorded. The absence of an adequate response of the myocardium and its vascular supply to an increase in volume load in rats with cardiac hypertrophy secondary to increased pressure load suggests, however that a relative myocardial ischaemia and cardiac insufficiency finally will develop in these animals.

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CHRONIC NON RHEUMATIC VALVULAR HEART DISEASE

An Autopsy Study

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Hallgrímsson, J. Chronic non-rheumatic valvular heart disease. An autopsy study. Acta path. microbiol. scand. Sect. A, 84 247-252, 1976.

The frequency of chronic non-rheumatic valvular heart disease in Iceland was investigated via autopsies performed from November 1963 through December 1974. During this period, about 12.400 Icelanders died at the age of 16 years and older and 28.8 per cent of these were included in the study. At autopsy males outnumbered females by 2:1. The frequency of calcific aortic stenosis was found to be 5.63 per cent and the prevalence was calculated to be 5.17 per cent among males and 4.50 per cent among females. Calcific aortic stenosis in tricuspid valves was more frequent in females and calcific aortic stenosis in bicuspid valves was more frequent in males. Among the hearts with calcific aortic stenosis, 70.8 per cent were found to have normally tricuspid valves, 25.4 per cent bicuspid valves and 3.8 per cent tricuspid valves with an anticommissural fusion. In 0.59 per cent of the hearts the aortic valve was either bicuspid or had an anticommissural fusion without the features of calcific stenosis. However a functional stenosis was suggested by the increased weight of most of these hearts. The frequency of bicuspid aortic valves was 1.2 per cent with a prevalence in males of 1.34 per cent and in females 0.50 per cent. A calcified mitral annulus was found in 1.90 per cent of the hearts and in most, it was either associated with calcific aortic stenosis in a tricuspid valve or it was a single valvular disease. Rheumatic valvular disease was found in 1.08 per cent of the hearts examined.

Key words: Non-rheumatic valvular heart disease; autopsy study; calcific aortic stenosis; congenital deformity of aortic valve; calcified mitral annulus.

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The frequency of chronic valvular heart disease among Icelanders 16 years of age and older was studied in a series of autopsies performed in the Department of Pathology at the University of Iceland. A paper on the observed cases of chronic rheumatic valvular heart disease has recently been published (8). This paper describes the other chronic valvular heart diseases found, i.e. congenital aortic valvular deformities, calcific aortic stenosis and calcified mitral annulus.

MATERIAL AND METHODS

The collection of the material presented here is described in detail in a recently published paper on chronic rheumatic valvular heart disease and therefore only a few facts will be repeated here (8).

From November of 1963 through December of

TABLE 1 *Aortic Valvular Diseases and Calcified Mitral Annulus in 3578 Hearts*

	Males	Females	Total	Per cent of all hearts
Calcific aortic stenosis	76	54	130	3 63
Tricuspid	45	47	92	2.57
Bicuspid	29	4	33	0.9?
Unicommusural fusion	2	3	5	0.14
Deformed aortic valve without calcific stenosis	17	4	21	0 59
Bicuspid	8	2	10	0.28
Unicommissural fusion	9	2	11	0.31
Calcified mitral annulus	22	49	71	1.98

TABLE 2. *Prevalence of Non-rheumatic Aortic Valvular Heart Disease in Iceland by Sex per Hundred*

	Males	Females
Calcific aortic stenosis in tricuspid valves	1.88	3.92
Calcific aortic stenosis in bicuspid valves	1.21	0.33
Calcific aortic stenosis, all types combined	3 17	4.30
Bicuspid aortic valves, with and without calcific stenosis	1.54	0 50

1974 about 12.400 Icelanders died at the age of 16 years and older and 3578 of these, corresponding to 28.8 per cent, were autopsied and the author examined the hearts. The sex ratio of the subjects autopsied during the period was 2 males to each female which means that about 40 per cent of the males and 25 per cent of the females who died in these age groups are included in the study.

The non-rheumatic diseases were diagnosed by the gross inspection and palpation of the heart valves. The pliability of the aortic valve cusps was tested with the fingers and was considered to be normal when the cusps fell easily against the aortic wall. The grade of stenosis was estimated from the observation of the valvular orifice and the rigidity of the fibrotic and calcified cusps. The calcification of the mitral annulus was detected by palpation and transverse cuts through the valve base and annulus.

RESULTS

Table 1 shows the observed non-rheumatic valvular diseases. The bicuspid aortic valves, whether associated with calcific stenosis or not, were all of the common type with two free commissures and two cusps unequal in size the larger with a raphe extending from near the center of the cusp across the sinus of Valsalva to the aortic wall. The valves with unicommissural fusion had three cusps of equal size and two of the cusps were adherent for a length varying from 0.5 to 0.8 cm.

In 92 hearts, the calcific aortic stenosis involved an otherwise normally formed tricuspid valve. In 38 hearts, the calcific aortic stenosis was superimposed on deformed valves, i.e. bicuspid valves and valves with an unicommissural fusion. Most of the bicuspid valves in this group were in males. Twenty-one hearts had bicuspid or unicommissurally fused aortic valves without fibrosis and calcification, i.e. the cusps were normally thin and pliable. Males outnumbered females in both groups of deformities.

In Table 2 the unequal representation of the sexes in the material is taken into account. Calcific aortic stenosis in tricuspid valves was more common in females and calcific aortic stenosis in bicuspid valves was more common in males. The prevalence of calcific aortic stenosis, all types combined, was higher in females. The prevalence of

TABLE 3 *Morphological Grades of Two Types of Calcific Aortic Stenosis*

	Males	Females	Total
Tricuspid valve			
Mild	23	23	46
Moderate	12	9	21
Severe	6	10	16
Uncertain	4	5	9
Bicuspid and unicommissurally fused valves			
Mild	12	5	17
Moderate	2		2
Severe	4	1	5
Uncertain	13	1	14

bicuspid aortic valves was three times as high in males as in females.

The aortic stenosis was mild in about 70 per cent of the hearts in which such an evaluation was attempted (Table 3). Most of the moderately and severely stenotic valves were tricuspid. The stenosis was not graded in a little over one-third of the aortic valves with pre-existing deformities which may account for the low numbers of moderately and severely stenotic valves in that group.

The average weight of the hearts was higher with each grade of increasing calcific aortic stenosis (Table 4). In the group of hearts with deformed aortic valves but without calcific stenosis the average weight was above normal in both sexes which suggests that most of these aortic valves were functionally stenotic although they felt soft to palpation. Five hearts only in the latter group, all in males, weighed less than 360 grams.

Table 5 shows that the mean age at death was higher among the patients with calcific aortic stenosis superimposed on a normally tricuspid valve than it was among the patients with calcific stenosis superimposed on deformed valves. The lowest mean age at death was observed among the patients with deformed valves without calcific stenosis. The oldest patients were those with a calcified mitral annulus.

Eight patients with aortic valvular disease, or 5.3 per cent, had a past medical history of rheumatic fever. Four of them had calcified

TABLE 4 *Average Weight of Hearts in Grams by Types of Disease, Grades of Stenosis and Sex*

	Males	Females
Calcific aortic stenosis in tricuspid valve		
Mild	497	430
Moderate	517	437
Severe	631	520
Calcific aortic stenosis in bicuspid and unicommissurally fused valves		
Mild	502	360
Moderate	515	
Severe	657	633
Bicuspid and unicommissurally fused aortic valves without calcific stenosis	438	415
Calcified mitral annulus only	433	368

TABLE 5 *Age Distribution and Mean Age at Death by Types of Valvular Disease in Both Sexes*

	Years								
	25–34	35–44	45–54	55–64	65–74	75–84	85–94	95–	Mean age
Calcific aortic stenosis of tricuspid valve									
males				3	11	19	12		78
females				2	12	19	12	2	80
Calcific aortic stenosis of deformed valves									
males		2	2	2	12	12	1		70
females		1			5		3		73
Deformed aortic valves without calcific stenosis									
males	2	1	3	3	6	2			59
females		1			2	1			64
Calcified mitral annulus, isolated									
males					2	2	4		80
females					1	12	8		82

tricuspid valves, three had unicommissural fusions in the valves, two of which were calcified and one patient had a calcified bicuspid valve. These 8 patients were from 64 to 78 years old at death and all had only had a single episode of arthritis at the age of 20 or younger without a recurrence or subsequent heart symptoms until a few years before death. The criteria set up for the diagnosis of rheumatic disease were not present in the hearts of these patients (8).

A grossly calcified mitral annulus was observed in 71 hearts, 22 being males and 49 females (Table 1). Most of them occurred either in association with calcific aortic stenosis in tricuspid valves or were an isolated valvular disease and none were observed in hearts with deformed aortic valves without calcification (Table 6). Among the 38 hearts with calcific stenosis superimposed on deformed aortic valves were only 5, or 14 per cent, with a calcified mitral annulus, but

TABLE 6 *Aortic Valves in Hearts with Focal and Diffuse Calcification of the Mitral Annulus*

	Focal calcification of mitral annulus		Diffuse calcification of mitral annulus		Total
	Males	Females	Males	Females	
Calcific aortic stenosis in tricuspid valve	9	12	2	14	37
Calcific aortic stenosis in deformed valves	2	1	1	1	5
Deformed aortic valves without calcific stenosis					
Normal aortic valve	1	3	7	18	29

among the 92 hearts with calcific stenosis superimposed on tricuspid valves were 37 or 40 per cent, with a calcified mitral annulus.

DISCUSSION

According to *Roberts* the true incidence of valvular aortic stenosis in elderly persons is unknown and one of his reasons for this statement was that mild degrees of obstruction may be overlooked at autopsy (16). In this study all grades of valvular aortic stenosis were included and the frequency was found to be 3.63 per cent which means that it is a common disease. This percentage is considered representative of the population in Iceland, since 40 per cent of the males and 25 per cent of the females who died in a period of over 9 years are included. Hudson considered aortic valve calcification with age to be frequent but not causing severe stenosis he quoted *Dry & Williams* who found 23 cases among 2616 autopsies, or 0.9 per cent (9).

In Iceland, calcific aortic stenosis is more frequent in females which confirms *Roberts* findings in a series of elderly patients (16). *Hultgren* found the frequency to be equal in the sexes (10) but *Hudson & Campbell* found it to be higher in males (4, 9).

The bicuspid aortic valve is generally considered to be the most common congenital malformation of the heart and it occurs more frequently in males than in females or about 4 to 1 (2, 7). Forty-three bicuspid aortic valves were observed in the 3578 hearts examined, or 1.2 per cent with a male/female ratio of 6/1. *Roberts* estimated the frequency of the bicuspid aortic valve with and without calcific stenosis, to approach 2 per cent (13). *Bacon & Mathews* quoted a much lower incidence based on data supplied by 7 authors, viz. 0.54 per cent (2). The results of such pooling from various sources is likely to differ from those found in individually planned prospective studies.

Among the hearts with calcific aortic stenosis in this series were 33 or 25.4 per cent, with bicuspid valves, which is in the range of *Campbell's* results of 15–36 per cent (4). These figures differ from those obtained by *Roberts* who reported that bicuspid valves occurred in over 50 per cent of patients with anatomically isolated aortic stenosis (15, 16). The difference may lie in the mode of selection of the material in these three studies.

Sixteen hearts in this series had an unicommissural fusion in the aortic valve. In 5 of these there was an associated calcific stenosis. *Roberts* found 7 such valves in 21 hearts with calcific aortic stenosis (16). The fusion is congenital in some instances and acquired in others and it can be difficult or impossible to distinguish between the two (13).

The bicuspid aortic valve is anatomically imperfect and therefore more prone to suffer from stress which leads to degeneration and subsequent calcification (5, 6). Calcific aortic stenosis therefore occurs at an earlier age in such valves than it does in normally formed tricuspid aortic valves where wear and tear is considered to be the sole cause (4). The unicommissurally fused aortic valve is probably also malfunctioning and therefore more likely to suffer early from degeneration and calcification (1, 3). This is confirmed here (Table 5).

Some bicuspid and unicommissurally fused valves escape degenerative and calcific changes and function through a normal life span. It is likely that such valves are better formed and therefore less strained than the valves of the same types which become sclerotic. An individual difference in resistance may also exist as people develop calcific stenosis at different ages and most escape entirely although it is said that microscopical degenerative changes can be found in nearly all heart valves in old age. Thus 10 of the 43 bicuspid valves and 11 of the 16 valves with an unicommissural fusion were grossly thin and felt normally pliable. Microscopical examination of these valves might have disclosed degenerative alterations. The increased weight of most of these non-sclerotic hearts suggests that the deformities were causing a mild stenosis (Table 4).

In this series, almost 3 out of every 4

calcified stenotic aortic valves were normally tricuspid and without commissural fusion. Among 60 stenotic aortic valves, *Campbell* found 40 to be tricuspid (4) *Rapaport* & *Roberts* on the other hand concluded in their studies that most calcified stenotic aortic valves were bicuspid and *Roberts* pointed out in his paper that mild degrees of obstruction in the aortic valve may be overlooked at autopsy (11 15) Bicuspid valves are less likely than tricuspid valves to be passed unnoticed by the pathologist, which can explain the discrepancy between these studies. Thus about 50 per cent of the stenotic valves observed here were graded as mild.

Roberts found at autopsies on patients with calcific aortic stenosis that there was a difference in the structure of the aortic valve in old and young patients. Thus, in patients 65 years of age and older the aortic valve was tricuspid in 90 per cent and bicuspid in 10 per cent. In patients between 16 and 65 years of age the aortic valve was tricuspid in 45 per cent, congenitally bicuspid in 45 per cent and congenitally unicuspid in 10 per cent (15 16) These findings are confirmed here. Seventy three per cent of the patients 66 years of age and older with calcific aortic stenosis had tricuspid valves, 22 per cent had bicuspid valves and 5 per cent had unicommissurally adherent valves. In the patients between 16 and 66 years of age the aortic valve was tricuspid in 50 per cent and bicuspid in 50 per cent The 5 patients with commissural fusion and calcific aortic stenosis were all in the older age group here but *Roberts* reported that commissural fusion was unusual in old patients and common in the young (16)

The frequent association of a tricuspid calcified stenotic aortic valve and a calcified mitral annulus is explained by the common aetiology of wear and tear with age The less frequent association of a calcified stenotic bicuspid aortic valve with a calcified mitral annulus is explained by the younger age of the patients and the premature degeneration of the malfunctioning bicuspid aortic valve (Table 6)

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LIVER BIOPSIES FROM PSORIATICS RELATED TO METHOTREXATE THERAPY

1 Findings in 123 Consecutive Non-Methotrexate Treated Patients

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Nyfors, A. & Poulsen, H. Liver biopsies from psoriatics related to methotrexate therapy 1 Findings in 123 consecutive non-methotrexate treated patients. Acta path. microbiol. scand. Sect. A, 84 253–261 1976

A prospective study was started in 1969 to describe morphological features of liver biopsies from patients with severe psoriasis. Among 123 patients evaluated for possible MTX therapy liver biopsies disclosed pathological histology (mainly fatty change and/or non-specific reactive hepatitis) in 31 per cent. The incidence of pathological liver histology did not statistically correlate with psoriasis parameters such as duration and extent. However, statistically significant correlations ($p<0.0001$) were found between the frequency of pathological liver histology and other factors such as age, obesity and daily alcoholic intake. Comparison of liver histology with SGOT values at the time of liver biopsy showed that while the diagnostic specificity of this test was high (1.00) the diagnostic sensitivity was low (0.17)). Normal values of SGOT should not be relied upon to indicate all types of liver pathology. A "risk index" indicating the probability of pathological liver histology was developed. It is calculated as follows: two times the height (cm) minus weight (kg) minus age (years) minus 50 (in case of daily alcoholic intake) minus 50 (in case of elevated SGOT). To elucidate liver histology and particularly to rule out fibrosis and cirrhosis, a liver biopsy should be performed in every psoriatic patient with a low score in the risk index prior to beginning MTX therapy.

Key words: Liver biopsies; histology; psoriasis; risk index.

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In 1951 Gubner (9) noted that psoriasis vulgaris disappeared from patients with rheumatoid arthritis during treatment with oral doses of the folic acid antagonist aminopterin. In 1953 a new and less toxic folic acid antagonist, amethopterin (Methotrexate) was marketed in the United States. Methotrexate (MTX) was then used to an increasing extent for treatment of the most severe cases of psoriasis. MTX therapy in single weekly oral doses of 25–35 mg has been reported to clear psoriasis vulgaris in 60–70 per cent of patients (4, 15). During recent years, however, an increasing number of cases of liver disease, particularly cirrhosis, have been reported in psoriatics during MTX therapy (1, 3, 5, 6, 8, 14, 16, 17, 18, 19, 22, 26, 27, 28, 30).

In early 1969 little information about the

TABLE 2 *Summary of Laboratory Tests in Relation to Liver Histology*

Laboratory tests	*Pathological* (59)									*Normal* (57)
	Liver histology of 116 successful liver biopsies. The figures indicate number of patients with abnormal laboratory tests.									
	cirrhosis	fibrosis	mixed changes		non-specific reactive hepatitis	fatty changes			Total	
			mild	moderate		mild	moderate	severe		
Serum aspartate aminotransferase	1	1	1	0	1	2	3	1	10	0
Alkalinephosphatase	1	1	0	0	1	0	0	0	3	1
Serum bilirubin	1	0	0	0	1	0	0	0	2	0
Serum albumin	1	0	0	1	1	0	0	0	3	6
Serum gammaglobulin	1	1	1	1	3	11	6	4	28	17
Normotest	0	0	0	0	1	0	0	0	1	3
Platelet count	1	0	0	0	0	0	0	0	1	2
ESR	0	1	0	1	2	2	3	3	12	8
Haemoglobin	0	0	0	0	0	1	1	0	2	3
Leucocytes	0	0	1	1	1	1	1	0	5	2
Serum creatinine	0	0	1	0	0	0	1	0	2	1

Mixed changes: non-specific reactive hepatitis and fatty change

TABLE 3 *Chief Histological Diagnoses in 123 Liver Biopsies*

	Number	Per cent of diagnosed
Cirrhosis	1	0.9
Portal fibrosis and alcoholic hepatitis	1	0.9
Mixed changes (non-specific reactive hepatitis and fatty change)	6	5.1
Non-specific reactive hepatitis	8	6.9
Fatty change	43	37.1
Normal	57	49.1
		Sum 100.0
Unsuccessful	7	

Psoriatic arthropathy in 12 patients (7 males, 5 females) had been treated with phenylbutazone given within 1 year of liver biopsy to 5 patients, and up until the time of liver biopsy to 4 patients.

Hepatomegaly was detected in 1 patient (liver biopsy cirrhosis) but no patients appeared to have ascites.

The main clinical findings of psoriasis are listed in Table 1.

Among 123 patients, 122 had psoriasis vulgaris. Only one patient had nail psoriasis as the sole manifestation of psoriasis. Of the 122 patients with *psoriasis vulgaris*, 25 had psoriatic arthropathy and 79 had additional nail psoriasis. No patient had arthropathy as the only manifestation of psoriasis. All patients with arthropathic psoriasis were seronegative (Latex test). In 8 patients the psoriasis covered more than 80 per cent of the skin surface. The mean involvement of the skin surface with psoriasis for all patients studied was approximately 32 per cent (range 2–98 per cent) with 38 per cent (range 3–98 per cent) for males and 25 per cent (range 0–93 per cent) for females. Visible regions of the face and hands were involved with psoriasis in 97 patients.

Laboratory Findings

Table 2 reports the chief results of laboratory tests at the time of initial liver biopsy.

Histological Findings

Table 3 presents the chief histological diagnoses in the 123 liver biopsies. The degree of mixed changes included 4 mild, 1 moderate, and 1 moderate with epitheloid cell granulomas. The 8 non-specific reactive hepatitis biopsies were of mild degree, while the 43 biopsies with fatty changes were assessed as follows: 26 mild, 12 moderate and 5 severe. A total of 59 of 116 successful liver biopsies were pathological, which is 51 per cent (95 per cent confidence limits 42–61).

Correlations

By discriminant analysis the following variables were found to be relevant to the chief histological diagnoses: age, overweight, daily alcoholic intake and elevated SGOT. In the light of these significant correlations, an index indicating the risk of occurrence of pathological liver histology was developed. The index was calculated to be the sum of the following: 2 times the height (cm) minus weight (kg) minus age (years) minus 50 (in case of daily alcoholic intake) and minus 50 (in case of elevated SGOT). A *low index* score indicate a high risk of pathological liver histology.

Table 4 shows the correlations between this index and the chief histological diagnoses. The correlation was found to be highly statistically significant: chi-square 33.9, DF 4, $p < 0.0001$.

TABLE 4 *Correlations between the Risk Index and the Chief Histological Diagnoses*

Score in risk index	Liver histology number of patients with						
	No information	cirrhosis fibrosis	mixed changes	non-specific reactive hepatitis	fatty change	Normal	Sum
90–99	0	0	0	0	1	0	1
100–109	0	1	0	0	1	0	2
110–119	0	1	0	0	0	0	1
120–129	0	0	0	0	3	0	3
130–139	0	0	0	1	2	0	3
140–149	1	0	0	0	3	2	6
150–159	2	0	2	0	4	1	9
160–169	2	0	0	0	9	2	13
170–179	1	0	2	1	7	1	12
180–189	0	0	1	1	2	3	7
190–199	0	0	0	1	4	8	13
200–209	0	0	1	1	4	8	14
210–219	0	0	0	2	0	6	8
220–229	0	0	0	0	2	9	11
230–239	1	0	0	1	1	8	11
240–249	0	0	0	0	0	7	7
250–259	0	0	0	0	0	1	1
260–269	0	0	0	0	0	0	0
270–279	0	0	0	0	0	1	1
Sum	7	2	6	8	43	57	123

Mixed changes non-specific reactive hepatitis and fatty change.

No statistical significant correlations were found between the chief diagnoses of liver histology and the following variables duration and extent of psoriasis vulgaris, sleep-disturbing itching, onycholysis, previous intake of systemic corticosteroids or phenylbutazone or arsenic or other possible hepatotoxic medicines, previous gallstones, diabetes mellitus or pregnancy

DISCUSSION

When this prospective study of the histological features of liver biopsies from middle aged patients with severe psoriasis was started in 1969 few papers on the subject were available (12)

Since that time several reports (Table 5) have been published on the histological features of liver biopsies from psoriatics.

The Co-operative Study (28) had the largest series of liver biopsies, while the study of *Zachariae & Søgaard* (29) should be emphasized because of controls. In the other studies the number of pathological liver biopsies is too small to allow statistical comparison between liver pathology psoriasis parameters and other variables.

In both studies mentioned above and in the present study the criteria for selection of patients have been virtually the same The histological evaluation of liver biopsies has also been conducted in the same manner (10)

Amongst the differences the Co-operative Study had significantly more patients previously treated with systemic corticosteroids (23 per cent against 10.5 per cent) Patients in the Co-operative Study also had a statistically significant larger area of the skin sur

TABLE 5 *Pathological Findings in Different Studies of Liver Histology in Patients with Severe Psoriasis Considered for Methotrexate Therapy*

Author	Number of abnormal liver biopsies from a total of	Per cent	Number of fibrosis	Per cent	Number of cirrhosis	Per cent
Co-operative Study (28)	approximately 2/3–200	66	45	22.5	3	1.5
Zachariae (29)	approximately 2/3– 47	66	3	6.0	0	0.0
Reese (21)	29– 35	83	1	3.0	0	0.0
Berge (3)	16– 32	50	0	0.0	2	6.3
Almeyda (1)	14– 25	56	4	16.0	0	0.0
Tobias (26)	18– 19	95	8	42.0	0	0.0
Palmer (19)	8– 14	57	0	0.0	0	0.0
Roenigk (22)	7– 13	54	1	8.0	0	0.0
Dahl (6)	8– 8	100	1	12.5	0	0.0
Podurgiel (20)	3– 6	50	0	0.0	0	0.0
Average		*68*		*11.0*		*0.8*
Nyfors (present study)	59–116	51	1	0.8	1	0.8

face involved with psoriasis (62 against 32 per cent). A possible explanation is that physicians in the United States, from which 57.2 per cent of liver biopsies in the Co-operative Study came, have been more cautious about starting MTX therapy and have instead tried systemic corticosteroids. At the Finsen Institute systemic corticosteroids are avoided if possible due to the risks of side-effects and exacerbations of psoriasis when treatment is stopped (rebound phenomenon).

The Co-operative Study did not find a statistically significant correlation between liver pathology and age, sex, or duration of psoriasis. *Zachariae & Sogaard* (29) did not find overweight to be significantly correlated with liver pathology.

The 3 studies agree closely in sex and age distribution and alcoholic intake. The duration of psoriasis was also equal in the study of *Zachariae & Sogaard* (29) and in our study.

An essential finding in our study is the 51 per cent frequency of pathological changes in liver biopsies. This result agrees well with the other reports (Table 5). Our most frequent findings were fatty change and non-specific reactive hepatitis, which correspond well with findings in other studies (3, 28, 29).

Realization of the fact that more than half of the patients with severe psoriasis considered for MTX therapy have pathological liver histology raises 2 questions:

1. Is the frequency of pathological liver histology higher in psoriatics than in normal controls of approximately the same age?

2. If yes, could the high frequency of fatty change and/or non-specific reactive hepatitis be related to psoriasis or other factors such as age, overweight, alcoholic intake or treatment of psoriasis?

Only a few studies of liver histology in controls in the same age group are available (11, 29).

Hilden et al. (11) found fatty change in 24 per cent of 503 persons who had died in traffic accidents, with a statistically significant correlation between the frequency of fatty change and age. Fatty change was seen mainly over the age of forty.

Zachariae & Sogaard (29) found pathological liver histology in 67 per cent of 60 people (average age 61 years) who had died from Parkinson's disease, heart disease or traffic accidents.

These 2 studies of control groups and the

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failure to find a statistically significant correlation between liver histology and psoriasis parameters in the present study seem to indicate that liver pathology is more dependent on factors such as age, overweight and alcoholic intake, than on psoriasis.

Our results (Table 3 and 4) indicate that abnormal liver histology particularly fibrosis and cirrhosis is to be expected in patients with a low score in the risk index described in this paper Below a certain score, such as 150 the probability of pathological liver histology is high, whereas such probability is low above a certain value of the index, such as 210 With values of the risk index between 150–210 pathological findings can be expected in about half of the patients.

It seems logical that psoriatics with a low score in the risk index should have a liver biopsy performed prior to initiating MTX therapy since one immediately would expect that patients with pre-existing liver disease tolerate MTX less well than patients with a normal liver

Other results of this study indicate that the sensitivity of biochemical tests such as the SGOT (0 17) was too low to reveal fatty change and non-specific reactive hepatitis. Furthermore clinical examination of the patients gave little information about possible liver disease.

CONCLUSIONS

1 Pathological liver histology (mainly fatty change and/or non-specific reactive hepatitis) occurred in 51 per cent of patients with severe psoriasis considered for MTX therapy
2. These pathological findings appeared to be connected with factors such as age, overweight, and alcoholic intake to a far greater extent than with psoriasis.
3 The probability of occurrence of pathological liver histology in psoriatics considered for MTX therapy was found to be statistically correlated with age, overweight, daily alcoholic intake and elevated SGOT
4 A risk index for the probability of occurrence of pathological liver histology is presented. It is calculated as follows twice height (cm) minus weight (kg) minus age (years) minus 50 (in case of daily alcoholic intake) and minus 50 (in case of elevated SGOT)
5 To elucidate liver histology and to particularly rule out fibrosis and cirrhosis, a liver biopsy is recommended in patients with a low score in the risk index before beginning MTX therapy
6. The diagnostic sensitivity of the SGOT was found to be low (0 17) indicating that normal values of this test are unreliable indicators of fatty change and non-specific reactive hepatitis.

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LIVER BIOPSIES FROM PSORIATICS RELATED TO METHOTREXATE THERAPY

2. Findings before and after Methotrexate Therapy in 88 Patients. A Blind Study

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Nyfors, A. & Poulsen, H. Liver biopsies from psoriatics related to methotrexate therapy. 2. Findings before and after methotrexate therapy in 88 patients. A blind study. Acta path. microbiol. scand. Sect. A, *84*: 262–270, 1976.

Eightyeight patients with severe, recalcitrant psoriasis had liver biopsies performed before and after Methotrexate (MTX) therapy. MTX was given for an average of 26 months as a single, weekly, oral dose of 25 mg maximum. The mean cumulative dose was 1735 mg (range 175–4590 mg). A statistically significant increase in the number of pathological post-MTX liver biopsies was found ($p < 0.0001$). Of the 88 patients 6 developed cirrhosis and another 5 developed fibrosis, in all 12.5 per cent, during MTX therapy (95 per cent confidence limits for cirrhosis: 3–14 per cent). There was no statistically significant correlation between the number of pathological post-MTX liver biopsy findings in the 88 patients and the following variables one by one: cumulative dose of MTX, duration of MTX therapy and admitted alcoholic intake during MTX therapy. Cirrhosis and fibrosis did not develop statistically more frequently from pathological than normal pre-MTX liver histology ($p = 0.062$). The liver damage appeared to be due to a multifactorial interaction of straining factors on the liver during MTX therapy. A multifactorial index comprising: cumulative dose of MTX, admitted alcoholic intake during MTX therapy, age, obesity and, if available, pre-MTX liver histology gave an estimate of the probability of developing cirrhosis or fibrosis during treatment of psoriasis with weekly, oral doses of MTX. For use of MTX therapy in psoriasis the following precautions are suggested: MTX therapy should be used only in disabling cases, a pre-MTX liver biopsy and repeat liver biopsies at regular intervals of ½–1 year should be performed, alcohol should be prohibited and frequent inquiries should be made about the patient's alcoholic intake and strong reliance should not be placed on the SGOT as an indicator of abnormal liver histology.

Key words: Methotrexate therapy; psoriatics; liver biopsies; histology; risk indexes.

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Case reports of liver disease, particularly cirrhosis, observed in patients with psoriasis during Methotrexate (MTX) therapy have aroused great concern (3, ref.).

Prospective studies including both pre-MTX liver biopsies and post-MTX liver biopsies from the same patients with psoriasis are few, and most often include a small number of patients with short follow-up periods and low total doses of MTX.

The purposes of this study were to evaluate

and compare histological findings in liver biopsies from patients with severe psoriasis before and after MTX therapy and to correlate possible changes in liver histology with MTX therapy

MATERIAL AND METHODS

Selection of Patients

Patients included in this study must have had

1. Typical and severe psoriasis unresponsive to previous treatment.
2. At least two liver biopsies including the initial one which were more than 5 mm long
3. Willingness to cooperate.
4. No evidence of cirrhosis or fibrosis in the pre MTX liver biopsy
5. MTX given in a single weekly oral dose of 25 mg maximum.

Material

Of the original 123 patients 35 (3) did not fulfil these requirements. Among patients excluded from the study 7 had unsuccessful pre MTX liver biopsies, 3 died prior to the post MTX liver biopsies, 23 would not cooperate and 2 had cirrhosis or fibrosis in the pre-MTX liver biopsies.

The present material comprises 88 pre-MTX liver biopsies and 117 post MTX liver biopsies from the same 88 patients. Post MTX liver biopsies were performed once in 65 patients, twice in 17 patients, and three times in 6 patients. Histological findings in the latest liver biopsy of each patient were used for comparison with each pre MTX liver biopsy

Methods

MTX therapy was usually started within 2 days following the pre MTX liver biopsy with an initial dose of 25 mg of MTX taken orally. The patients were warned to avoid alcohol and to decrease the risk of drug interactions by not taking the following drugs acetylsalicylic acid containing analgetics, barbiturates thiazides, sulphonamides and other possible hepatotoxic medications (3 ref)

During MTX therapy the following blood tests were done four to six times a year SGOT alkaline phosphatase serum billirubin, serum gamma globulin, serum creatinine sedimentation rate and haemoglobin. Possible side-effects of MTX, cumulative and actual doses of MTX and admitted alcoholic intake were also noted (3 ref)

All liver biopsies were performed using the Menghini technique

The histological evaluation of the liver biopsies was done blindly without knowledge of which liver biopsies came from pre MTX or post MTX treatment periods. To make the histological study as controlled as possible, liver biopsies from random patients with other skin diseases than psoriasis were intermingled A strict system of histological evaluation of liver biopsies was utilized, as described elsewhere (3)

Statistics

The significance of difference in the frequencies of pathological findings in the post MTX liver biopsies was evaluated by Fisher's Exact test and Mann-Whitney U test (3 ref.)

In order to evaluate the factors contributing to the formation of pathological findings in post MTX liver biopsies two indices were constructed. Index I can be used in patients who have not had a pre therapy liver biopsy The components were MTX cumulative dose, admitted alcoholic intake during MTX therapy age and obesity These factors were given points as follows: 1) MTX cumulative dose 0–1000 mg 1 point, 1001–2000 mg 2 points, 2001–3000 mg 3 points, more than 3000 mg 4 points, 2) alcoholic intake under 3 drinks daily 2 points 1–3 drinks daily 3 points, more than 3 drinks daily 4 points, 3) age: 0–49 years zero points, 50–69 years 1 point, older than 70 years 2 points, 4) obesity present 1 point, absent zero points (3 ref)

Index II was designed to be used in studies of MTX treated psoriatics with a pre MTX liver biopsy and comprised the 4 factors mentioned above plus the chief histological diagnoses of the pre-MTX liver biopsy The diagnoses were given the following scores: cirrhosis/possible cirrhosis/fibrosis 4 points, mixed changes 3 points, nonspecific reactive hepatitis 2 points fatty change 1 point and normal zero points.

The scores from the different factors in index I and II of the 88 patients were tabulated (Table 8 and 9)

The significance of difference between patients with scores of 2–4 and 5–8 respectively was evaluated by the Fisher's Exact test and Mann-Whitney U test (3 ref)

RESULTS

Clinical findings (before MTX therapy) pertinent to liver histopathology

A complete survey of the clinical aspects of psoriasis in these patients is published elsewhere (3 ref) The mean age of the 42 males and the 46 females was 50 years (range 21 78 years)

Table 1 (left column) shows the admitted alcoholic intake before MTX therapy

TABLE 1 *Admitted Alcoholic Intake before and during Methotrexate Therapy in 88 Psoriatics*

Alcoholic intake	Before methotrexate	During methotrexate
Occasionally	46	56
1–3 drinks a week	12	23
1–3 drinks a day	22	6
More than 3 drinks a day	8	3
Total	88	88

TABLE 3 *Number of Normal and Pathological Liver Biopsies before and after Methotrexate Therapy*

	Before methotrexate	After methotrexate
Normal	47	28
Pathological	41	60
Total	88	88

TABLE 2 *Results of Laboratory Tests before and during Methotrexate Therapy in 88 Patients*

Laboratory tests	Two days before first liver biopsy Number of abnormal tests	During methotrexate therapy Number of patients with abnormal values	Two days before latest liver biopsy Number of abnormal tests
Serum aspartate aminotransferase (~ SGOT)	2	60	4
Serum bilirubin	0	2	1
Alkaline-phosphatase	0	9	4
Blood platelets	2	17	2
Serum albumin	4	24	6
Serum gamma globulin	32	73	48

TABLE 4 *Chief Pathological Findings in Liver Biopsies from 88 Psoriatics before and after Methotrexate Therapy*

Diagnosis		Before methotrexate		After methotrexate	
Cirrhosis			0		5
Possible cirrhosis			0		1
Fibrosis	moderate	0	0	1	5
	mild	0		4	
Mixed changes	moderate	1	3	2	5
	mild	2		3	
Non-specific reactive hepatitis	moderate	0	6	1	7
	mild	6		6	
Fatty change	severe	3	32	2	37
	moderate	7		7	
	mild	22		28	
Total			41		60

Mixed changes: non-specific reactive hepatitis and fatty change.

Potentially hepatotoxic medicine had been used by 25 patients (13 males, 12 females) and included estrogens 7 chlorpromazine 4 gold salts 3, chlorothiazide 3, penicillin 5 tetracycline 2 and methyldopa 1

Jaundice had previously occurred in 13 patients, while 9 had gall-stones verified by cholecystography and 2 diabetes mellitus. Twentyeight patients were obese. No patients had neither hepatomegaly nor ascites.

Laboratory Findings

The chief results of laboratory tests performed prior to liver biopsies and during MTX therapy are presented in Table 2. Elevations in serum gamma globulin often tended to last for longer periods, e.g. during winter while the other tests were only elevated occasionally No differences were found between the two sexes.

Liver Histology

Table 3 shows the number of normal and pathological liver biopsies before and after MTX therapy

Table 4 indicates the chief pathological findings in liver biopsies before and after MTX therapy One liver biopsy contained epithelioid cell granulomas both before and after MTX therapy

Due to the strict entrance requirements for patients into this study no cases of cirrhosis, possible cirrhosis, or fibrosis were present in pre MTX liver biopsies. In contrast, after MTX therapy there were five cases of cirrhosis, including one case each with additional mild fatty change, mild mixed changes (fatty change and non-specific reactive hepatitis) moderate fatty change and moderate fatty change with alcoholic hepatitis In addition, findings included one case of possible cirrhosis with moderate fatty change and five cases of portal fibrosis, of which four had mixed changes (1 severe 2 moderate and 1 mild) and one had mild non-specific reactive hepatitis.

Table 5 presents the relati[illegible] tatus of liver histology in pre and post MTX [illegible] opsies All five sets of liver biopsies with decreased changes originally showed fatty change which either decreased or disappeared. The 45 sets of liver biopsies with unchanged histology included 26 normal sets, 17 sets with fatty change and two sets with non-specific reactive hepatitis.

TABLE 5 *Comparison of Pathological Changes between the Latest Liver Biopsy after Methotre ate and the Li[illegible] Biopsy before Methotrexate in each of 88 Patients*

Decreased liver pathology	5
Unchanged liver histology	45
Increased liver pathology	38

Despite findings of normal histology fatty change and/or non-specific reactive hepatitis in the pre-MTX liver biopsies, fibrosis or cirrhosis did develop in 11 patients during MTX therapy Table 6 shows pre and post MTX liver histology with pertinent clinical features from these 11 patients. This group had an average age of 57 years. Their admitted alcoholic intake during MTX therapy is specified in Table 6 while the admitted alcoholic intake for all 88 patients is shown in Table 1 (right column) Eight of the 11 patients with cirrhosis/fibrosis had a normal SGOT at the time of liver biopsy (Table 6)

MTX Therapy

The average duration of MTX therapy at the time of the latest liver biopsy was 26 months, with a range of 2–72 months in the 88 patients. During MTX therapy the weekly oral dose had been given continuously in 44 patients. The 44 remaining patients had had interruptions in therapy of more than a month. Most patients had been treated approximately 80 per cent of the time (range 7–100 per cent)

The average cumulative dose of MTX at the time of the latest liver biopsy in the 88 patients was 1733 mg (range 175–4590 mg) The mean cumulative dose of MTX in the 11 patients who developed cirrhosis or fibrosis was 2158 mg (range 525–4590 mg)

TABLE 6. *Features from the 11 Patients with Cirrhosis or Fibrosis*

Patient number	Chief histologic diagnosis pre-MTX	Chief histologic diagnosis post MTX	Time between biopsies (year)	Age and sex	Ethanol intake before MTX	Ethanol intake during MTX	MTX total dose (mg)	SGOT at last biopsy
365	moderate fatty change	cirrhosis	1.5	44 M	d	b	925	elevated
228	mild mixed changes	cirrhosis	2.2	56 M	d	b	1395	normal
237	normal	cirrhosis	2.3	71 M	b	c	1405	elevated
294	moderate fatty change	cirrhosis	3.3	57 F	a	a	3010	normal
227	mild fatty change	cirrhosis	4.8	68 M	c	b	2253	elevated
345	mild mixed changes	possible cirrhosis	2.0	50 M	d	d	525	normal
260	mild non-specific reactive hepatitis	fibrosis	3.5	46 F	a	a	3588	normal
235	normal	fibrosis	3.7	71 M	a	a	845	normal
295	mild fatty change	fibrosis	3.8	62 F	a	a	2819	normal
100	moderate mixed changes	fibrosis	4.0	53 M	c	a	2165	normal
210	normal	fibrosis	4.5	52 M	b	a	4590	normal

a occasionally b 1–3 drinks a week c 1–3 drinks a day d more than 3 drinks a day

TABLE 7 *Association between Chief Histological Diagnoses before and after MTX Therapy*

Pre-MTX histology		Post MTX histology				
Diagnosis	Total	Cirrhosis/fibrosis	Mixed changes	Non-specific reactive hepatitis	Fatty change	Normal
Cirrhosis/fibrosis	0	0	0	0	0	0
Mixed changes	3	3	0	0	0	0
Non-specific reactive hepatitis	6	1	1	2		0*
Fatty change	32	4	3	0	23	2*
Normal	47	3	0	6	12	26
Total	88	11	4	8	37	28

* $p<0.05$, Sign test.

The clinical efficacy of MTX therapy in psoriasis and the side-effects attributable to MTX therapy from the 88 psoriatics are described elsewhere (3 ref.) In this study 86 of 88 patients improved, with an average decrease of psoriasis from 37 to 13 per cent of the body area covered, while only two patients were unchanged 0-86 $p<0.01$ Sign test.

TABLE 8. *Chief Histological Diagnoses of 88 Post Methotrexate Liver Biopsies Related to Score in Index I (MTX Cumulative Dose, Alcoholic Intake during MTX Therapy, Age and Obesity)*

	Post-methotrexate liver histology					
Score in Index I	Cirrhosis/fibrosis	Mixed changes	Non-specific reactive hepatitis	Fatty change	Normal	Total (number of patients)
2–4	1	3	6	24	22	56
5–8	10	1	2	13	6	32

$p<0.01$ Fisher's Exact test.

Correlations

There are obvious differences between the histology of the pre- and post-MTX liver biopsies (Table 5). Table 7 shows the association between the histological diagnoses before and after MTX therapy with respect to the statistical comparison.

Of 47 patients with normal biopsies before MTX therapy, 21 developed pathological liver histology during MTX therapy.

Of 41 patients with abnormal liver histology before MTX therapy, two were histologically normal after MTX therapy and 39 remained pathological. The frequencies of 21/47 and 2/41 are significantly different, $p<0.0001$.

Cirrhosis, possible cirrhosis and fibrosis were found in pre- and post-MTX liver biopsies with respective frequencies of 0/88 and 11/88 (12.5 per cent, 95 per cent confidence limits 6–21). Since no patients with cirrhosis or fibrosis in the pre-MTX liver biopsies were allowed into this study, no statistical tests of significance could be applied to the above frequencies.

No statistically significant difference was found between development of increased histological changes from normal or abnormal pre-MTX liver biopsies. Increased changes developed in 21/47 patients with normal initial biopsies and 17/41 patients with pathological initial biopsies. Although cirrhosis and fibrosis developed more frequently in patients with abnormal pre-MTX liver biopsies (8/41 patients) than in patients with normal pre-MTX biopsies (3/47), the difference is not statistically significant (Fisher Exact test, $p = 0.062$) at the 95 per cent level of significance.

There was also no statistically significant correlation between the number of pathological post-MTX liver biopsy findings and any of the following variables separately: cumulative dose of MTX, duration of MTX therapy, obesity and admitted alcoholic intake during MTX therapy.

The 11 patients developing cirrhosis or fibrosis during MTX therapy did not have a statistically significant higher cumulative dose of MTX than the 28 patients whose liver histology remained normal during MTX treatment (Mann-Whitney U test, $p = 0.19$). The 11 patients also did not have a significantly higher alcoholic intake during therapy than the 28 patients ($p>0.05$).

Consideration of the supposed pathogenetic factors on the liver showed increasing changes in the liver histology as the score in the indices increased. If the 88 patients, for instance, were divided into 2 groups based on their scores in index I, 1 out of 56 patients with a score of 2–4 developed cirrhosis, while 10 out of 32 patients with a score of 5–8 developed cirrhosis/fibrosis ($p<0.01$) (Table 8). If the patients with normal pre-MTX liver histology were divided into the same 2 groups based on index I, none of the 34 with scores of 2–4 and 3 of 13 patients with scores of 5–8 developed cirrhosis/fibrosis (Fisher's Exact test, $p = 0.017$).

Index II showed an even more pronounced correlation ($p<0.001$) between a high score in the index and development of pathological liver histology during MTX therapy. If the

TABLE 9 *Chief Histological Diagnoses from 88 Post-Methotrexate Liver Biopsies Related to Score in Index II (Pre-MTX Liver Histology, MTX Cumulative Dose, Alcoholic Intake during MTX Therapy, Age and Obesity)*

Score in Index II	Post-methotrexate liver histology					Total (number of patients)
	Cirrhosis/fibrosis	Mixed changes	Non-specific reactive hepatitis	Fatty change	Normal	
2–4	1	1	4	13	21	40
5–8	10	3	4	24	7	48

* $p<0.01$ Fisher's Exact test.

patients again were divided into 2 groups with a score of 2–4 and 5–8 based on index II one of 40 and 10 of 48 patients developed cirrhosis/fibrosis (Fisher's Exact test $p = 0.0088$) (Table 9)

DISCUSSION

Pathological findings in liver biopsies after MTX therapy for leukemia have been reported by several authors. In 1955 *Colsky et al* described fibrosis in post mortem liver specimens of three MTX treated leukemic children. The cumulative dose of MTX varied from 237 to 1057 mg over a 6–9 months treatment period MTX was given periodically in daily doses of 2.5 to 10 mg *Hutter et al.* (1960) found no fibrosis in 18 "normal" children who died suddenly but fibrosis in 31 per cent of 26 untreated leukemic children and fibrosis in 80 per cent of 247 leukemic children treated with cytostatic drugs such as MTX or prednisone

Several reports on MTX hepatotoxicity in patients with non-malignant diseases have been published. Most attribute the liver changes to MTX with documentation of abnormal liver function tests or pathological liver biopsy findings following MTX treatment. Few reports, however document increasing liver pathology between pre MTX and post MTX liver biopsies in the same patient. This should be required for concrete documentation of MTX hepatotoxicity as liver function tests have a limited value in demonstrating pathological liver histology (3, ref)

A Co-operative study using MTX treated patients with psoriasis (3 ref) included 38 sets of liver biopsies from the same patient before and after MTX therapy and found a statistically significant increase in the degree of fatty change ($p<0.02$) and fibrosis ($p<0.05$) In contrast *Zachariae et al.* (1975) (3 ref) *Warin et al* (1975) (3 ref) *Reese et al.* (1974) (3 ref) and *Almeyda et al.* (1972) (3 ref) found no statistically significant difference between pre and post MTX liver biopsy findings in 56, 25 21 and 9 psoriatic patients respectively

Other authors compare liver biopsy findings in untreated psoriatic patients with post MTX liver biopsy findings in other psoriatics, thus preventing patients from serving as their own controls. *Zachariae & Søgaard* (1973) (3 ref) found no statistically significant difference between pre MTX liver histology in 47 psoriatic patients and post MTX liver histology in 40 other psoriatic patients. *Reese et al.* (1974) (3 ref) came to the same conclusion in a similar comparison between two groups of 35 psoriatic patients each.

Several authors have examined post MTX liver biopsies and attempted to correlate histological findings with MTX dosage route of administration and duration of treatment (Table 10) Cirrhosis was found in an average of 10 per cent and fibrosis in 20 per cent of patients. The frequency of cirrhosis and fibrosis was highest in patients who had taken MTX in small, daily doses (3 ref)

TABLE 10. *Post Methotrexate Liver Biopsy Findings in Psoriatics in Literature*

Author	MTX route oral (P.O.)/ intramuscular (I.M.)	Liver biopsies: number	Liver biopsies: cirrhosis	Liver biopsies: fibrosis	Suspected relationship to: cumulative	Suspected relationship to: dosage frequency	Suspected relationship to: ethanol
Tobias & Auerbach	P.O. + I.M.	69	5 (7 %)	22 (32 %)	Yes	?	?
Dahl et al.	P.O. + I.M.	44	6 (14 %)	11 (25 %)	?	Yes p = 0.05	No
Almeyda et al.	P.O. + I.M.	42	3 (7 %)	12 (29 %)	Yes cirrhosis + fibrosis p = 0.05	N	?
Roenigk et al.	P.O.	37	6 (16 %)	2 (5 %)	No	No	N
Podurgiel et al.	P.O.	35	4 (11 %)	4 (11 %)	No	Yes p<0.01	?
Reese et al.	P.O.	33	1 (3 %)	2 (6 %)	No	No	Yes fatty change p<0.001
Palmer	P.O.	23	3 (13 %)	4 (17 %	No	No	?
Nyfors & Poulsen (19)	P.O.	88	6 (7 %)	5 (6 %)	No	No	No

and was lowest in patients receiving MTX at weekly or longer intervals (5 ref.). No statistically significant correlation was found between pathological liver histology findings and the total dose of MTX, except by *Almeyda et al.* (1972) (3, ref.) in patients with cirrhosis and fibrosis (p = 0.05). *Podurgiel et al.* (1973) (3 ref.) found that the frequency of MTX intake per month correlated significantly with liver biopsy changes (p< 0.01). This agrees with *Dahl et al.* (1971) (3, ref.) who found cirrhosis and fibrosis occurring more frequently in psoriatics receiving oral MTX in small, daily doses than in those receiving larger weekly doses of MTX. *Palmer* (1973) (3 ref.) found cirrhosis and fibrosis in 30 per cent of 23 psoriatics given small, daily doses of MTX.

Several authors found that concomitant use of alcohol by psoriatic patients during MTX therapy increased the pathologic changes in liver histology (Table 8). The amount of alcohol consumed always remains uncertain, as the admitted intake has often been found later to be considerably less than the real amount of alcoholic intake.

The present study demonstrated a statistically significant increase in the number of pathological findings in liver biopsies during MTX therapy. It was not possible to show a statistically significant correlation between increasing liver changes in the 88 patients and the following factors one by one: cumulative dose of MTX, duration of MTX therapy, admitted alcoholic intake during MTX therapy or obesity. However multifactorial evaluation (by means of the described indices) of the probability of liver damage during MTX therapy demonstrated a highly statistically significant association between the number of patients with cirrhosis or fibrosis and the following factors grouped together: pathological pre MTX liver histology, a high total dose of MTX, regular daily alcoholic intake during MTX therapy, advanced age, and obesity. The 11 patients who

developed cirrhosis, possible cirrhosis, fibrosis, diagnosed from 1.5 to 4.8 years after starting MTX therapy are of particular interest. There was a tendency (p = 0.062) for cirrhosis and fibrosis to develop more frequently in patients with pathological rather than normal pre-MTX liver biopsies. The propriety of using the 95 per cent level of significance, rather than the 90 or 80 per cent level can be questioned, as a lower significance level might seem more appropriate to decrease the probability of development of cirrhosis and fibrosis. The three men in this study who had cirrhosis or possible cirrhosis diagnosed within the first three years of MTX therapy had relatively low cumulative doses of MTX, but later admitted to large alcoholic intakes (more than 4 drinks a day). Alcohol consumption thus seemed to be a likely factor contributing to development of cirrhosis. In agreement with this one of these men had the histologic picture of alcoholic hepatitis in addition to cirrhosis.

SGOT was of little value in revealing liver pathology as 8 of 11 patients with cirrhosis/fibrosis had normal SGOT value at the time of post MTX liver biopsies (95 per cent confidence limits 73 per cent, 39–94 per cent).

The justification of liver biopsy in psoriatics before MTX therapy has been discussed elsewhere (3). The need for a liver biopsy during MTX therapy can seemingly be evaluated using the described indices. Index I should be used in studies of psoriatics in MTX therapy without a pre-MTX liver biopsy while index II should be used in similar studies with a pre MTX liver biopsy. A high score in these indices indicates increasing risk of cirrhosis/fibrosis.

CONCLUSIONS

Comparison between pre and post MTX liver biopsies has shown 1) a statistically significant increase in the number of pathological post MTX liver biopsies ($p < 0.0001$) 2) a tendency ($p = 0.062$) towards more frequent development of cirrhosis and fibrosis from pathological than normal pre-MTX liver biopsies, and 3) a highly statistically significant more frequent development of cirrhosis or fibrosis in patients with combinations of the following features: pathological pre MTX liver histology, a high total dose of MTX, daily alcoholic intake during MTX therapy, advanced age and obesity. Of 88 patients, 11 developed cirrhosis or fibrosis during MTX therapy.

Since there are patients with recalcitrant psoriasis who require MTX, we advocate the following:

1. MTX should be used only in disabling psoriasis.
2. Performance of a pre MTX liver biopsy and repeat liver biopsies at regular ½–1 year intervals.
3. Prohibition of alcoholic intake with frequent inquiries about alcoholic intake.
4. Limitation of reliance on the SGOT as an indicator of abnormal liver histology.
5. Use of the above mentioned indices to evaluate the probability of multifactorial damage to the liver during MTX therapy.

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BILE CANALICULAR ALKALINE PHOSPHATASE AND DISEASE

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Hägerstrand, I. Bile canalicular alkaline phosphatase and disease. Acta path. microbiol. scand. Sect. A, 84: 271–277, 1976.

The alkaline phosphatase reaction is normally absent in human bile canaliculi, but was found in 79 patients. In search for a common causal factor these patients were further examined. Thirty-seven were autopsied. The conditions most commonly associated with the phenomenon were malignant tumours with or without involvement of the liver, collagen diseases, long standing partial obstruction of the common bile duct, and genetic variants of alpha-1-antitrypsin. No clinical or laboratory facts were common to all the patients.

Key words: Liver; enzymology; phosphatases.

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In the healthy human liver, the alkaline phosphatase activity is histochemically localized to endothelial cells of sinusoids and large vessels and, in various liver diseases, this activity increases. Appreciable activity of the bile canaliculi was demonstrable in 14 per cent of liver biopsy specimens, one third of which showed involvement of malignant tumours (8). This work is concerned with an analysis of the patients with canalicular alkaline phosphatase activity without tumour involvement of the liver biopsy specimens.

MATERIAL AND METHODS

In the period from 1967 to 1973 altogether 870 biopsy specimens of the liver were obtained—mainly by Menghini's needle—from patients in General Hospital, Malmö, Sweden. One hundred and two specimens showed normal histology and histochemistry. Intense and extensive alkaline phosphatase activity along the bile canaliculi was revealed in 38 specimens presenting tumour involvement, in 79 without signs of tumour. The latter specimens, deriving from 79 patients, were used in the present investigation.—As regards treatment of the tissue and the histochemical method used, reference is made to no. 8 in the list of references.

The patients were classified into 4 groups according to liver histology and sinusoidal enzyme activity (Table 1). The number of women and men in the groups was roughly equal, and the age distribution curves in the different groups were similar (range 17–85 years). Out of 38 patients (16 women, 22 men) who had died, 37 had been autopsied. The series of 46 patients with abnormal liver histology included 5 with cirrhosis, 4 with hepatitis (1 sarcoidosis) and 1 with amyloidosis; the changes in the remaining patients were mild: mild cholestasis, steatosis or fibrosis.

The clinical records and autopsy records applying to the patients were examined. The follow-up was continued until the end of 1974.

Plasma protein analyses were performed according to *Laurell et al.* (15).

Serum alkaline phosphatase (3.1.3.1) activity was determined according to a modification of the method used by *Bessey et al.* (3), the normal range in the case of adults being 2–8 units (mean ± 2 S.D.).

Iso-enzymes of serum alkaline phosphatase were studied by way of electrophoresis in agarose gel

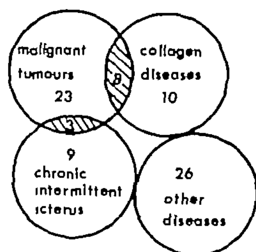


Fig. 1. The distribution of diagnoses over 79 patients with diffusely extended and intense canalicular alkaline phosphatase reaction observed in liver biopsy specimens.

origin and extent were found. Nine of 27 autopsied patients with tumours had no liver metastases, in another 4 metastases to the liver were few and small (<2 cm). As regards the 7 patients with tumours who survived, symptoms of liver metastases did not occur; laparatomy had been performed on some of these. Patients without metastases to the liver were more common in groups I and III (normal sinusoidal alkaline phosphatase activity) than in groups II and IV (increased sinusoidal alkaline phosphatase activity), 11 and 5 subjects, respectively. As regards the 27 autopsied patients, 6 had shown bone metastases and 6 pulmonary metastases. The following tumours were found: carcinomas of the pancreas (4), the gallbladder (1), the common bile duct (1), the liver (hepatocellular 1), the stomach (4), the large intestine (2), the prostatic gland (4, 3 of which were latent cancers), the bronchial tree (4), the mammary gland (5), the kidney (3), the uterine cervix (1), the thyroid (2), insuloma (1), reticulocellular sarcoma (1), malignant lymphogranulomatosis (2) and myeloproliferative syndrome (2). —If the 38 subjects with tumour growth in the liver biopsy specimens were added (see under Material and Methods), 117 (14 per cent) out of 820 examined liver biopsy specimens showed extensive and intense canalicular alkaline phosphatase reaction. 72 (61 per cent) of these specimens derived from subjects with tumour diseases.

In the series of 79 patients, 18 (22 per cent) had signs of collagen diseases, in 8 associated with malignant tumours. Six of the latter patients were in group I. In 5 of the tumour patients a paramalignant type of muscular and arthritic pain was manifest. The remaining collagen diseases included rheumatoid arthritis, lupus erythematosus, Takayasu's arteritis, periarteritis nodosa, temporal arteritis, scleroderma.—Nine patients (11 per cent) had choledocholithiasis. In these patients intermittent icterus and increased serum alkaline phosphatase had been manifest for some months. Some of the patients had been subjected to surgery on several occasions on account of choledocholithiasis. Among patients with tumours, 3 with carcinoma of the pancreas exhibited the same clinical picture. Eleven of the 12 patients with icterus belonged to group IV and also had increased sinusoidal alkaline phosphatase activity.

The remaining subjects were suffering from various diseases: hepatitis associated with alcohol abuse, contraceptive pills, abortion or development into chronic active liver disease (4 subjects), cirrhosis associated with genetic variants of alpha 1-antitrypsin or alcohol abuse (4), steatosis associated with alcohol abuse (1), thrombosis of the portal vein associated with barbiturate intake indicated by epilepsy (1), hepatocellular necroses due to Bilivistan® (2), heart failure (4), pulmonary embolism (1), pulmonary tuberculosis (1), generalized sarcoidosis (1), sepsis (2), regional enteritis (1), obscure diagnoses (4).

What regards the autopsied patients, excluding those with hepatic metastases and cirrhosis, the liver was usually of normal form, size and appearance. Three livers were diffusely enlarged and rather pale but without appreciable steatosis (liver weights 2760, 2050, 2100 g; body weights 71, 55, 44 kg, respectively).

DISCUSSION

No common causal factor responsible for the increase in alkaline phosphatase of the human bile canaliculi was found. The total biopsy material included patients with malignant tumours and collagen diseases without canalicular alkaline phosphatase in the liver specimens, but we do not know the ratios between the patients with and without this activity. The liver biopsy specimens presenting increased canalicular alkaline phosphatase activity were in 60 per cent derived from patients with malignant tumours, with or without metastases to the liver.

Most of the patients included in the present study had received drugs and some of the latter might have influenced the canalicular alkaline phosphatase, and some patients were alcohol abusers (1 9 12). Any single drug was not regularly given to the patients. The findings do not allow any conclusions.

The frequency of the Z-gene of alpha-1 antitrypsin in the 79 patients comprised in this study was high (9 individuals) 2.5 times higher than that observed in individuals in an unselected Swedish population (5 16). The frequency among the patients of the total biopsy material is unknown, but it is not improbable that the frequency in such a selected material is higher than in the unselected population. Globules found to be positive in periodic acid Schiff (= PAS) staining after diastase treatment (23) were seen only in 10 of the 820 specimens, corresponding to 1.2 per cent as compared with an expected frequency of 5 per cent with regard to the Z-gene frequency (5 16).

As regards the laboratory findings, a high sedimentation rate, an electrophoretic pattern of an inflammatory reaction without any increase in immunoglobulins, and a moderate increase in serum alkaline phosphatase and gamma-glutamyl transferase activities was often found to co-exist with an increased alkaline phosphatase of the bile canaliculi, while thrombocytosis, increased activities of aminotransferases, hepatomegaly and mild bilirubinaemia were less regular findings.— The canalicular alkaline phosphatase activity showed no sex differences and occurred irrespective of blood groups.— On the basis of the present investigation, little can be deduced about the persistence or variation of the canalicular activity since biopsy was not repeated. Persistent activity was shown only in one patient at autopsy. He died with active disease one year after the liver biopsy had been obtained. In some patients, however *serum* alkaline phosphatase normalized after operation of a tumour or after treatment with cytostatics and hormones.

A serum iso-enzyme of alkaline phosphatase has been reported to appear in connection with combined hepatic and pulmonary diseases as well as in connection with obstructive and neoplastic hepatic disease (15 18) and abuse of alcohol (4). Thus, the conditions under which this type of alkaline phosphatase activity has been found to occur in serum are similar to those in which we have found a canalicular activity of the enzyme (2 9). This iso-enzyme migrates slowly or not at all, on starch gel or polyacrylamide disc gel electrophoresis, but fast (migrating with the alpha-1-globulins of serum) in cellulose acetate (Cellogel®) (13 14). The main fraction of human bile alkaline phosphatase shows the same migration qualities (13 14 19) and the alkaline phosphatase activity localized to the bile canaliculi seems to correspond to this bile and serum iso-enzyme. Twelve patients comprised in the present series were studied with respect to serum iso-enzymes. However the discussed fraction could not be separated by agarose gel electrophoresis.

Different malignant tumours have been reported to produce alkaline phosphatase such as the Regan iso-enzyme (7) its properties being similar to the placental iso-enzyme. Such an explanation of the increased canalicular alkaline phosphatase activity suggests that the enzyme produced in tumour tissue is secreted with the bile but it does not explain the increase in conditions other than tumour diseases.

In an earlier investigation (10) it was found that leucylaminopeptidase and gamma

glutamyl transferase activities often are histochemically increased in canaliculi in the presence of canalicular alkaline phosphatase activity On the basis of literature studies it was claimed also that it suggested a hepatic origin of all three enzymes, probably connected with induction of the hydroxylating enzymes of the smooth endoplasmic reticulum. Drugs and genetic variants of alpha-1-antitrypsin were discussed in relation to this hypothesis (10) Other findings in this investigation, suggestive of a connection with processes in the endoplasmic reticulum, include the common increase of acute phase reactants and biliary stasis. Acute phase reactants, including alpha 1-antitrypsin, are produced in the rough endoplasmic reticulum of hepatocytes and are secreted via the smooth reticulum. In biliary obstruction, morphological changes occur in the smooth reticulum and the hydroxylating reactions of the bile acids are changed (21 22) However the reason why canalicular alkaline phosphatase is prune to increase in longstanding, partial biliary obstruction remains obscure.—The findings of hepatomegaly in some patients in scintiscans or at autopsy might be consistent with a weight increase secondary to the induction processes (20)

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BILE CANALICULAR ALKALINE PHOSPHATASE IN NECROPSY SPECIMENS OF THE LIVER AND ITS RELATION TO DISEASE

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Hägerstrand, I. Bile canalicular alkaline phosphatase in necropsy specimens of the liver and its relation to disease. Acta path. microbiol. scand. Sect. A, 84: 278–284, 1976.

The histochemical patterns of the alkaline phosphatase reaction in liver specimens obtained at 185 consecutive autopsies were studied for any correlation with clinical data and post mortem findings. Alkaline phosphatase activity of bile canaliculi was found in 71 per cent of the subjects with malignant tumours not involving the liver and in 77 per cent of the subjects with malignant tumours involving the liver. The histochemical pattern did not differ with the type of tumour. Most subjects with rheumatoid arthritis as well as most of those with centrolobular hepatic necrosis due to heart incompensation also showed alkaline phosphatase activity in the bile canaliculi.

Key words: Liver; enzymology; phosphatases.

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The rather rare occurrence of alkaline phosphatase activity of bile canaliculi in human liver biopsy specimens was established in a previous study (4). Diseases found to be associated with the canalicular phosphatase activity were malignant tumours with and without liver engagement, collagen diseases and chronic, partial obstruction of the common bile duct (5). An abnormally high frequency of the Pi Z gene predisposing to alpha 1-antitrypsin deficiency was found among patients with canalicular alkaline phosphatase. Drugs and alcohol might have increased the canalicular activity in some individuals. The present study concerns the frequency of canalicular alkaline phosphatase activity in an unselected autopsy series, its relation to the diseases of the patients and a comparison with the panorama of the diseases of subjects without such activity.

MATERIAL AND METHODS

From 200 consecutive autopsies (performed at the Pathological Institute, General Hospital, Malmö, Sweden, in the autumn of 1974) a specimen (about 1.5 × 1 × 0.5 cm) was taken from the right, ventral surface of the liver 1–2 cm under the capsule. Care was taken not to include malignant tissue in the specimens. The material was obtained 3–50 hours after death of the patient. The cadavers had been kept at +4° C. The liver specimens were frozen in propane cooled by liquid nitrogen, and were preserved at −70° C until [illegible] fixed and stained for alkaline phosphatase activity as described elsewhere (4).

Paraffin-embedded liver specimens were studied in sections stained with haematoxylin-erythrosin as

well as with periodic-acid-Schiff (PAS) according to Mac Manus after diastase digestion. Clinical and autoptical records were analysed.

Plasma protein analyses were performed according to *Laurell et al.* (6). *Serum alkaline phosphatase* (3.1.3.1) activity was determined applying a modification of the method of *Bessey et al.* (2). In adults, the normal range was 2–8 units (mean ± 2 S.D.).

RESULTS

The Histochemical Findings and Their Relation to Disease

Among the 200 liver specimens, 185 were accepted for the study. The remaining 15 were split while frozen and when being cut. In the 185 specimens the alkaline phosphatase reaction product was distinct without signs of diffusion. There was no evident decrease or increase related to the interval between death and autopsy. Judging from the patterns of the reaction, 4 different groups could be recognized (Table 1. Fig. 1). The age distribution curve was equal in the groups, with a mean of about 70 years. The sexes were equally represented except in group IV which was made up of 20 women and 38 men.

Alkaline phosphatase reaction of the bile canaliculi was demonstrated in 58 per cent of the liver specimens, in 31 per cent with, in 27 per cent without an increased sinusoidal reaction.

The panorama of diseases is summarized in Table 2. The frequency of malignant diseases was 60 per cent, the frequency increasing

TABLE 1. *Histological Findings and Pattern of Alkaline Phosphatase Reaction. Four Groups (I–IV)*

Histochemical pattern	Group	Number of specimens
Normal endothelial reaction	I	47 (25 %)
Increased endothelial reaction	II	30 (16 %)
Increased canalicular reaction	III	50 (27 %)
Increased canalicular and endothelial reaction	IV	58 (31 %)

from group I (32 per cent) to group IV (88 per cent). The frequency of tumour involvement (metastases or primary) of the liver, of the lungs and of the skeleton in the different groups is given in Table 3. Of all the subjects with malignant tumours, 74 per cent were found to have canalicular alkaline phosphatase. The corresponding figure applying to those without liver involvement was 71 per cent, the figure applying to those with liver involvement being 77 per cent. The canalicular pattern was equally often observed together with a normal as with an increased sinusoidal reaction. Any correlation between the type or extent of the tumour and the presence or absence of canalicular alkaline phosphatase was not found, but metastases to the liver and lungs were found less frequently in subjects with a normal sinusoidal hepatic reaction.

Cardio- and cerebrovascular diseases were less frequent in the groups of patients with bile canalicular activity of alkaline phosphatase than in the groups of patients without such activity, and most subjects in groups III and IV in whom such diagnoses were established had centrolobular hepatic necroses due to heart incompensation.—Centrolobular hepatic necroses were found in 15 of the 185 specimens, 1, 10 and 4 in groups I, III, and IV, respectively. Two of the specimens showing necroses were obtained from patients in group III and one from a patient in group IV and originated from livers with multiple metastases. The only specimen with necroses without alkaline phosphatase activity of the bile canaliculi showed recent necroses due to pulmonary embolism, while the necroses of the other specimens were of various duration, sometimes with absorbed hepatocytes, but without healing.—Pulmonary diseases (tumours, embolism, pneumonia) were not overwhelming in the groups of patients with alkaline phosphatase activity of the bile canaliculi.

Among 10 subjects with collagen diseases (8 with rheumatoid arthritis without tumours, 1 with rheumatoid arthritis and a latent renal carcinoma, 1 with scleroderma and spreading


c
d

TABLE 2. *The Panorama of Diseases Found in the Different Groups*

Diagnoses	Groups I		II		III		IV	
Malignant tumours	15	(32 %)	14	(47 %)	31	(62 %)	51	(88 %)
Cardio- and cerebro-vascular diseases	27	(57 %)	13	(43 %)	13	(26 %)	7	(12 %)
Pulmonary embolism	9	(19 %)	9	(30 %)	11	(22 %)	18	(31 %)
Pneumonia	13	(27 %)	10	(33 %)	21	(42 %)	21	(36 %)
Collagen diseases	2		1		6		1	
Cirrhosis	0		2		1		3	
Icterus	0		5		0		3	
Cholecystolithiasis	17	(47 %)	7	(53 %)	18	(40 %)	21	(45 %)
Cholecysto- and choledocholithiasis	1		4		1		2	
Cholecystectomized	4		5		1		3	

TABLE 3. *The Extent of the Tumour Diseases Found in the Different Groups*

	Groups I	II	III	IV
Patients with malignant tumours	15 (32 %)	14 (47 %)	31 (6. %)	51 (88 %)
Patients with tumours of the liver	3 (20 %)	8 (57 %)	15 (50 %)	23 (48 %)
Patients with tumours of the lungs	3 (20 %)	5 (36 %)	13 (42 %)	24 (47 %)
Patients with tumours of the bone	4 (27 %)	2 (15 %)	8 (25 %)	18 (35 %)

renal carcinoma) 6 showed an isolated canalicular increase in alkaline phosphatase (including the subject with scleroderma and renal carcinoma, but not the subject with the latent renal carcinoma) None of the 10 subjects had received steroid therapy In 9 subjects, the collagen disease was active, in one patient (in group I) the rheumatoid arthritis was clinically inactive.

No group differences were demonstrable as regards gallstone disease Patients presenting a clinical picture of chronic, partial biliary obstruction due to gallstone were not observed.

Fig 1 Alkaline phosphatase patterns of liver tissue.

a. Normal enzyme reaction with staining along sinusoids next to portal zone (lower part) and around the central vein (upper right corner) Few positive canalicular structures are seen to the left. From a 56-years-old man with encephalomalacia and heterozygous for alpha-1 antitrypsin

b. Increased sinusoidal staining reaction and few visible canaliculi (lower part) Portal zone in the middle. From a 66-years-old man with spreading of both a renal and prostatic carcinoma.

c. An extensive canalicular positivity From a 32-years-old woman with intestinal leiomyosarcoma without liver involvement.

d. An extensive canalicular positivity From a 49-years-old man with myeloma and involvement of the pleural cavities.

Inclusion Bodies Characteristic of the Pi Z Gene Corresponding to Alpha 1 Antitrypsin Deficiency (5)

PAS-positive diastase resistant globules were found in 11 specimens (3.9 per cent 3 patients in group I II and IV 2 in group III) In 4 of the 11 cases, the electrophoretic pattern was consistent with that in heterozygous individuals as regards the Pi-genes.

TABLE 4 *The Serum Alkaline Phosphatase Values in the Different Groups*

Serum alkaline phosphatase	Groups I	II	III	IV
≦ 8	7 (58 %)	8 (53 %)	15 (60 %)	12 (44 %)
8–25	5 (42 %)	5 (33 %)	9 (36 %)	8 (29 %)
>25	0	2 (13 %)	1 (4 %)	7 (26 %)

The result of electrophoresis was the same in two other subjects (in group I) but their liver specimens contained no globules. Electrophoresis had been performed in 49 of the 185 subjects (11 7 16 and 15 in group I to IV respectively)

Serum alkaline phosphatase was determined in 79 subjects within the week preceding their death. The results are given in Table 4. Irrespective of the histochemical pattern, serum values had been normal in most subjects. The highest serum values were noted in patients in group IV in whom the activity in sinusoids as well as in canaliculi was increased.

The clinical records were carefully studied with a view to the *drugs* given. Different hormones and cytostatics had been given most frequently to subjects in group III and IV owing to the higher frequency of malignant diseases, but no particular substance was found to have been more regularly used in these groups than in the other 2 groups. Antihypertensive drugs and drugs against heart insufficiency some of which are known to cause liver lesions, were administered more frequently 40 subjects in groups I and II than to subjects in group III and IV Phenthiazines (Hibernal® Tindala®) and barbiturates were common drugs in all groups.—In about 5 cases abuse of alcohol was known from th past medical history but no patients were intoxicated at the time of admission.—Four patients had undergone operations 1 to 10 days before death In 3 of these subjects, an isolated canalicular increase of alkaline phosphatase reaction was shown, in 2 of these associated with centrolobular necroses of the liver parenchyma.

In an earlier study (5) hepatomegaly was found in subjects with alkaline phosphatase activity of the bile canaliculi. Therefore, the weights of the livers in patients in the various groups, excluding those with tumour growth, cirrhosis and advanced steatosis, were compared and related to the physical stature. It was not considered justified to use the body weights because subjects with tumours predominated in some groups, those with heart incompensation in some others The quotients liver weight/physical stature in patients in group I were not found to differ statistically significantly from those in patients in groups III and IV (normal alkaline phosphatase pattern compared with alkaline phosphatase increase of bile canaliculi and of bile canaliculi as well as sinusoids) but quotients in patients in group I were found to differ from those in group II (normal alkaline phosphatase pattern compared with isolated sinusoidal increased reaction $0.01 < p < 0.001$) quotients being highest in the latter group Canalicular activity of alkaline phosphatase without the mentioned associated conditions (tumour collagen disease centrolobular hepatic necrosis) was observed in 16 subjects. All of these presented heart incompensation complicated with pulmonary embolism or pneumonia. However identical combinations of diseases were equally common in subjects without the canalicular enzyme activity

DISCUSSION

Compared with the findings in studies of a liver biopsy material (5) the association between alkaline phosphatase activity of the bile canaliculi and malignant and collagen

diseases was confirmed in this autopsy study which, however, did not confirm an assumed connection between the canalicular enzyme activity and the Pi Z gene. In the autopsy material, centrolobular hepatic necroses proved to be associated with alkaline phosphatase activity of the bile canaliculi in the remaining parenchyma. Two specimens presenting the same histological picture were included in the biopsy material (5). In these, the necroses had most probably been caused by Biliviston® used at X-ray examination. The changed alkaline phosphatase pattern might be due to the necroses rather than to the drug *per se*. A functional overloading of the remaining parenchyma and/or the secondary regeneration may be considered as possible causes of the canalicular enzyme activity.—Any association between use of drugs or abuse of alcohol and the occurrence of canalicular alkaline phosphatase was not demonstrable.

The alkaline phosphatase activity of the bile canaliculi was more common in the material obtained at autopsy than in the biopsy material, 58 per cent and 14 per cent, respectively (4). This might be explained by the higher frequency of malignant diseases in the autopsy material where tumours were very common, 60 per cent, compared with 40 per cent as the normal figure applying to large autopsy series in the same population. In about 70 per cent of the subjects with malignant tumours, canalicular reaction was found to be increased irrespective of involvement of the liver (in 74 per cent in the total group of malignant tumours, in 71 per cent of those with tumours without involvement of the liver and in 77 per cent of those with tumours with such involvement). This finding obviously indicates a remote effect of the tumour on the liver.

No clear-cut connection between pulmonary diseases and the occurrence of canalicular alkaline phosphatase activity was found. Such a connection with the serum increase of the assumed canalicular isoenzyme has been reported (7). Any correlation between total serum alkaline phosphatase and the histochemical pattern of the enzyme in the liver was not demonstrable. It applies to all four histochemical patterns that a normal serum alkaline phosphatase was most common, while a large serum increase (> 25 E) was found mainly in subjects in whom both a canalicular and a sinusoidal increase was demonstrable by histochemical staining of the specimens. Three processes must be estimated when the histochemical pattern and the serum values are correlated: firstly, the cellular variations of enzyme activity, secondly, the escape of enzyme molecules from the membranes into the blood, and thirdly, the degradation of enzyme protein. The last process might also occur mainly in the liver (1). The three processes are not necessarily dependent on each other.

Isolated canalicular increase of alkaline phosphatase reaction was the common pattern of collagen diseases and centrolobular hepatic necroses, while tumour diseases without involvement of the liver might show both an isolated canalicular increase and a combined canalicular and sinusoidal increase. In the biopsy series (4), tumour diseases without liver involvement more often presented isolated canalicular enzyme increase.—In the necropsy series, the alkaline phosphatase activity of the bile canaliculi was not associated with hepatomegaly.

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VAST AND APPARENTLY PARADOXICAL CONTINUOUS RISE IN PLASMA RENIN AFTER REMOVAL OF GENTLY MANIPULATED SUBMAXILLARY GLANDS IN NEPHRECTOMIZED MICE

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Bing, J. & Poulsen, K. Vast and apparently paradoxical continuous rise in plasma renin after removal of gently manipulated submaxillary glands in nephrectomized mice. Acta path. microbiol. scand. Sect. A, 84 285–290 1976.

Gentle manipulation of the submaxillary glands of previously nephrectomized mice results in a vast increase in plasma renin concentration. The concentration reaches 1 to 7 G.U. renin/ml, which is 1,000-fold higher than normal plasma concentrations. The rise is much less pronounced if the glands are removed after the manipulation, confirming the site of the release. However, an apparent paradox is seen: the renin concentration continues to rise even after removal of submaxillary glands as well as kidneys, indicating that a bound form of renin is also released. The bound renin can be in the form of exocytosed granules or (and) in the form of "prorenin", the renin of which is activated with time. In spite of the very high renin concentrations the blood pressure is normal or only moderately increased. This disproportion between plasma renin and blood pressure can be caused by a simultaneous release of submaxillary kallikrein and renin, which have counteracting effects on the blood pressure.

Key words: Renin, plasma; submaxillary glands; nephrectomized mice.

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Werle et al. (1937) found that the submaxillary glands of mice contain large amounts of a renin-like pressor material. This finding has since been confirmed by various investigators who have studied the location and physiology of the submaxillary iso-renin (see *Page & McCubbin* 1968, *Takeda et al.* 1969, *Bing & Poulsen* 1971). Recently *Cohen et al.* (1972) were able to isolate in stable and pure form a family of potent hypertensive renin-like enzymes from the glands, and they have subsequently given a further characterization of pure submaxillary renin and used it for the development of a radioimmunoassay for direct measurement of renin (*Michelakis et al.* 1974 a and b). The present study was performed, because it was found that the manipulation necessary for submaxillary sialoadenectomy resulted in marked increase in plasma renin. Based on this finding the effect on plasma renin concentration of different

types of manipulation of the submaxillary glands was studied in previously nephrectomized mice. These studies were performed both with mice in which submaxillary sialoadenectomy was performed either immediately or some *minutes after the manipulations*, and with mice in which the glands were replaced after the manipulations.

MATERIAL AND METHODS

Animals male albino mice of the Danish Serum Institute strain, weighing about 35 to 50 g. Their mean total submaxillary renin content was well over 2,000 Goldblatt Units (G U) while the mean content of the two kidneys was about 40 G U (*Bing & Poulsen* 1975) The mice were pretreated with penicillin and bilaterally nephrectomized during a short-term ether anaesthesia about 17 hours before the experiments. On the day of the experiment they were again anaesthetized with ether and after a catheter had been placed in the femoral artery the first blood sample of about 20 µl blood was drawn from the catheter The remaining blood in the catheter was reinfused with a 5 per cent glucose solution containing 50 I U heparin/ml of which 0 12 ml/h was infused in order to avoid clotting. By the next samplings of about 20 µl blood the small volume in the catheter and the first µl blood were discarded.

Manipulation of the submaxillary glands was performed in different ways in some experiments the submaxillary glands were quickly lifted up from their normal position and thereafter either extirpated or replaced. In other experiments the glands were manually gently compressed once per

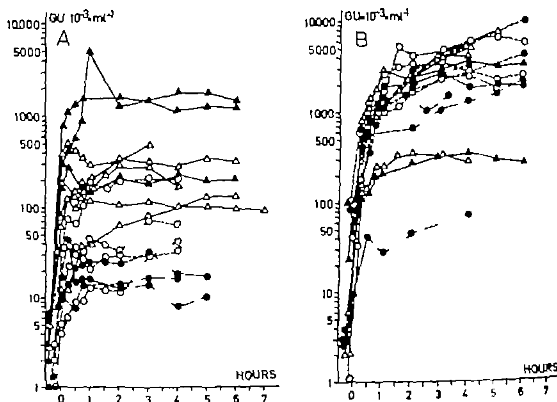


Fig 1 Effect on plasma renin concentration with time after different forms of manipulation of the submaxillary glands of previously nephrectomized mice While the glands were removed after the manipulation in some (Fig 1 A) they were replaced in other mice (Fig 1 B) The renin concentration in G U × 10 × ml is given on a logarithmic scale on the ordinates The abscissa shows the time in minutes and hours, the zero mark indicating the end of the manipulation. The symbol ⊘ marks experiments where sialo-adenectomy or replacement were performed immediately after the glands had been lifted up from their normal position. ○ marks manipulation for 0.5 to 1 minute, while and ▲ mark the experiments in which the glands after one minute gentle manipulation were removed (1 A) or replaced (1 B) after pause of 3 (△) or 6 (▲) minutes duration.

10 seconds to 30 to 60 seconds and thereafter either immediately or after a 3 or 6 minutes' pause removed or replaced. Immediately thereafter a second blood sample was taken and the anaesthesia stopped. Samples were thereafter taken first with 15 minute and later with 1 hour intervals from the now conscious mice, which were placed in restraining cages. In several of the mice the *blood pressure* was recorded using a Tybjerg-Hansen transducer and a Servogor 511 recorder. For comparison the blood pressure was followed in similarly nephrectomized sham-operated controls, in which the sham operation was performed as an incision on the abdominal wall in order not to touch the submaxillary glands.

Plasma renin concentration was determined using the capture radioimmunoassay for angiotensin I as described by *Poulsen & Jørgensen* (1974). The concentration was expressed in Goldblatt Units (G U) $\times 10^{-3} \times$ ml by comparison with the standard hog renin preparation obtained from Division for Biological Standards, Holly Hill, London.

RESULTS

I Plasma Renin Concentration after Gentle Manipulation of the Submaxillary Glands with Subsequent Removal of the Glands in Previously Nephrectomized Mice

The plasma renin concentration, which was between 1 and 7 G U $\times 10^{-} \times$ ml before the glands were touched, was about 10-fold increased immediately after the manipulation, when it only consisted in pulling the glands forward and ligating their vessels and ducts, and up to about 100-fold increased shortly after more pronounced manipulation (Fig. 1 A). Although kidneys as well as submaxillary glands had now been removed the renin concentration continued to rise steadily reaching a plateau after about one hour and most often staying at this level for the following up to 7 hours. The maximum level depended on the degree of manipulation being about 15 to 30 G U $\times 10 \times$ ml in 3 mice where the sialo-adenectomy was performed after the glands had been lifted up giving easy access to ligation of the vessels and the duct (●). In 3 of 4 mice in which the glands were repeatedly gently compressed for a half to one minute before they were removed (○) the level was between 30 and 60 while the fourth reached a level of about 200 G U $\times 10^{-3} \times$ ml^{-1} as shown in Fig 1 A, where the ordinate shows the renin concentration given on a logarithmic scale. In 8 mice in which one minute's manipulation was followed by a 3 minute (5 mice) or a 6 minute (3 mice) pause (△ and ▲ respectively) before the sialo-adenectomy was performed, the levels were still higher being from 100 to about 400 after 3 min pause and from 200 to 2000 G U $\times 10^{-3} \times$ ml^{-1} when the glands were removed 6 min after the end of 1 minute's manipulation (Fig. 1 A). This means that in the total material there was from about 15 up to about 2000-fold increase in plasma renin concentration.

II Plasma Renin Concentration after Gentle Manipulation of the Submaxillary Glands Followed by Replacement of the Glands in Previously Nephrectomized Mice

The results of the studies on the plasma renin concentration after gentle manipulation in previously nephrectomized mice in which the glands instead of being removed were replaced after the manipulation are given in Fig. 1 B in which the ordinate, as in Fig 1 A, gives the renin concentration in G U $\times 10^{-3} \times$ ml on a logarithmic scale. The figure shows that also here there is a steady increase in plasma renin, which is already markedly increased when the glands are replaced and thereafter steadily increases, most often reaching a level after about 1 hour. The results, however differ from those of the sialo-adenectomized mice in two respects: the first being that there is here no significant difference between the result of the different types of manipulation (immediate replacement in 7 mice (●), manipulation in 1–1½ min (○) in 3 mice and manipulation for 1 minute followed by a pause of 3 (△) to 6 (▲) minutes before replacement in 3 and 2 mice respectively. The second obvious difference is that most values reach higher levels (between 2000 and 7000 G U $\times 10 \times$ ml) than in the sialo-adenectomized mice.

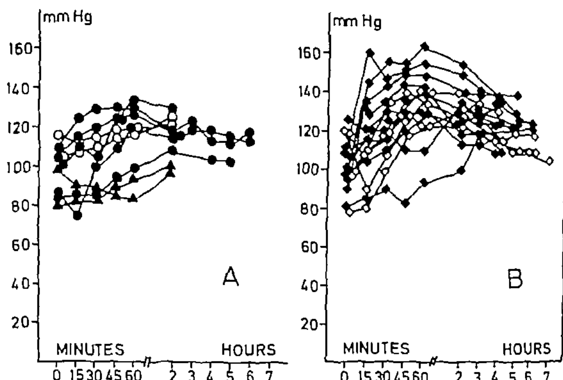


Fig 2 A shows the changes in blood pressure with time (given in minutes and hours on the abscissa) in 7 binephrectomized (● and ▲) and 2 submaxillary sialo-adenectomized as well as binephrectomized (○) mice The two mice marked ▲ had been anaesthetized 6 minutes longer than the other mice. The zero mark indicates the end of the anaesthesia.

Fig 2 B gives in a similar way the changes in blood pressure with time in 15 binephrectomized mice the submaxillary glands of which had been gently manipulated and thereafter replaced (◆) or removed (◇)

II Changes in Blood Pressure with Time fter Gentle Manipulation of the ubmaxillary Glands of Previously lephrectomized Mice

For comparison with the effect on the lood pressure of manipulation of the submaxillary glands the changes in blood pressure with time after the end of ether anaesthesia and sham operation were determined in 9 mice all of which had been binephrectomized about 17 hours before the experiment and 7 of which (● or ▲) had been sialo-adenectomized several days earlier The results are given in Fig 2 A. It shows that in most mice the blood pressure is lowest immediately after the end of the anaesthesia and thereafter rises to a plateau, which in 7 of the 9 mice is between 110 and 130 mm. The two mice, which had lower values (▲) had been anaesthetized for 6 minutes longer than the other mice. The blood pressures of these nephrectomized mice were as a whole a little lower than that found in 7 normal mice the values of which were between 115 and 140 mm.

Fig 2 B shows the effect of gentle manipulation of the submaxillary glands on the blood pressure of several of the mice the renin concentration of which is given in Fig 1 A and B. It is seen that the blood pressures here as in the controls (Fig 2 A) are low at the beginning of the curves, which marks the end of anaesthesia. From these values they rise to levels which are in the upper half or higher than those found in the controls. The values are as a whole higher after replace-

ment of the glands (10 mice, marked ♦) than after sialo-adenectomy (5 mice, marked ◇). With one exception the values do, however, not exceed 160 mm.

DISCUSSION

The vast increase in plasma renin concentration after gentle manipulation of the submaxillary glands of previously nephrectomized mice (Fig. 1 A and B) is probably caused partly by a nervous and partly by a physical mechanism. That a nervous mechanism plays a role is made probable by the finding that anaesthesia and binephrectomy can result in elevated plasma renin concentration, which is so prolonged that it cannot be due to release of renal renin during the nephrectomy (*Bing & Poulsen* 1975). The very much higher values found even after slight manipulation of the glands make it probable that also a physical factor plays a role. That the plasma renin concentration was steadily rising with time after removal of kidneys as well as submaxillary glands was an unexpected, paradoxically looking finding. It must be explained by a release of both free and bound renin, the bound renin being either in the form of granules, released by a form of exocytosis, or in the form of "prorenin" (for literature see *Leckie & McConnell* 1975) or by a simultaneous release of both forms of primarily inactive renin, which with time are activized in plasma. Such release of both active and inactive renin can also be the cause of the steady increase in plasma renin concentration in the mice in which the glands were replaced after the manipulation (Fig. 1 B). In most of these mice the renin concentration reaches values between 1 and 7 G U × ml^{-1}, which is about 1000-fold higher than the values found before the manipulation. The total amount of renin in plasma thus reaches about 18 G U., which is close to the amount found in one kidney but still only about one per cent of the well over 2000 G U. found as the mean of the content of the two submaxillary glands. While the degree of renin release to the blood, as could be expected, varied with the time between manipulation and removal of the glands in the sialo-adenectomized mice (Fig. 1 A), such relation was not seen in the mice, in which the glands were replaced at different times after the manipulation, the replacement allowing a continued and therefore also more pronounced release of renin.

Contrasting with the vast increases in plasma renin concentration are the unchanged or only moderate increase in blood pressure (Fig. 2 B) over that found in sham operated controls (Fig. 2 A). This lack of correspondence between renin concentration and blood pressure can be caused by a simultaneous release of kallikrein as well as renin, which are both found in the granules of the granulated ducts (*Chiang et al.* 1968). The blood pressures found are thus probably showing the combined result of the contrary actions of the hypertensive enzyme renin and the hypotensive enzyme kallikrein. It has so far not been examined if there is a depletion of renin substrate, which could be a further cause for the disproportion between plasma renin and blood pressure.

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POLYARTERITIS NODOSA ASSOCIATED WITH NOSEMATOSIS IN BLUE FOXES

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Nordstoga, K. & Westbye, Kr. Polyarteritis nodosa associated with nosematosis in blue foxes. Acta path. microbiol. scand. Sect. A, 84 291 296 1976.

The patho-morphological lesions in fox nosematosis (encephalitozoonosis) were studied in a material comprising 150 young blue foxes from 25 different farms. Disseminated nosematosis in blue fox pups was regularly accompanied by severe vasculitis, affecting medium-sized and small arteries in various organs. The acute damage has the form of a necrotizing angiitis, with mural necrosis and sometimes resultant thrombosis. The causative organism *Nosema cuniculi* is frequently present in the freshly affected arterial walls, either in endothelial or in medial smooth muscle cells. Older lesions include nodular fibrous thickening of the arterial walls, and intimal proliferation, sometimes with luminal obliteration. The conclusion is drawn that the arterial lesions are morphologically equivalent to classical polyarteritis nodosa.

Key words Polyarteritis nodosa Nosematosis blue foxes.

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Nosema cuniculi (syn. *Encephalitozoon cuniculi*) is a world wide protozoan parasite affecting a number of mammalian species, including man (1 3 4 8 9 11 15 16 18, 20, 21) Nosematosis is one of the most common infections in laboratory animals, and as it is often latent, the parasite may therefore disturb experimental studies. Taxonomically it has been classified as a microsporidia, thus being easily distinguished from other parasitic protozoes, such as toxoplasma by appropriate techniques (9 11 15 18) The parasite multiplies intracellularly until the parasitized cells, packed with spores, rupture and the spores are released into the surrounding tissue. In light microscopic sections the spores appear as elongate-ovoid bodies, measuring about 1.5 by 3.0 μ, with polar vacuoles which are of considerable diagnostic value. Other structures within the thin spore membrane include a polar filament which may be extruded under suitable conditions this process is considered as conclusive proof for the microsporidian character of the parasite (9) The spores do not stain well with haematoxylin-eosin, but are strongly Gram positive. Further staining characteristics have been given by others (9 11 15 16 18)

Nosematosis had not been known in Norway until 1968 when this infection was recognized in blue foxes (*Alopex lagopus*) During the last few years this parasite has caused heavy losses among blue fox pups in this country (15) A strange observation is that nearly all affected animals revealed vascular lesions consistent with classical polyarteritis nodosa, as cursorily reported in a previous paper (14) The aim of the present article is to describe the arterial lesions and to report

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the frequency of polyarteritis nodosa in fox nosematosis.

MATERIAL AND METHODS

Our material was collected during the years 1968–1974 and comprised a total of 150 young foxes from 23 different farms. The ages of most pups ranged from approximately 4 weeks to about 5 months. A majority of the animals, which were of both sexes, died from the infection, but the material includes also some animals which were killed in an exhausted condition.

Autopsies were performed according to routine schedules, and pieces of organs were normally fixed in a 10 per cent buffered solution of formaldehyde sometimes also in Carnoy's fixative. The material was embedded in paraffin and sectioned at about 5 μ the sections were routinely stained with haematoxylin and eosin (H&E) and a modified Gram method (16) The following additional stains were used on selected sections: Phosphotungstic acid haematoxylin, the acid picro-Mallory and Martius scarlet blue (MSB) methods (10) elastin van Gieson and methyl green-pyronin. Formalin fixed brain tissue was post-fixed in 2 per cent phosphate buffered osmic acid, dehydrated in acetone and embedded in Araldite Ultra thin sections were stained with uranyl acetate and lead citrate and examined in a Siemens Elmiscope 1 A.



Fig 1 Gross changes in coronary arteries the vessels are considerably thickened with prominent nodular lesions.

RESULTS

Autopsy Findings

In nearly all animals, the extra-mural coronary arteries exhibited alterations visible to the naked eye the vessels were variably thickened with nodular lesions resulting in prominent, distorted arteries (Fig 1) In a few animals the pericardium contained blood, or a fibrinous exudate The kidneys were frequently enlarged and pale sometimes with circumscribed spots on the external surfaces the spleen and the lymph nodes were often enlarged The meninges were hyperaemic and in a large number of animals there were haemorrhages in the meninges and the choroid plexus the lateral ventricles were frequently dilated and sometimes contained blood.



Fig 2 Arterial lesion with greatly thickened wall, infiltrated with inflammatory cells of various types. The homogeneous oval zone surrounding the narrowed lumen is stained as fibrin Myocardial artery MSB, × 100

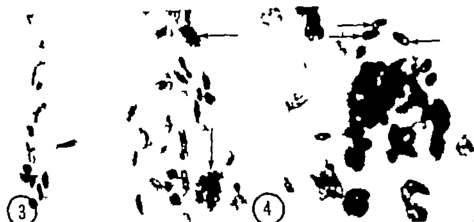


Fig. 3 Groups of *N. [illegible]* spores within the wall of a brain artery (arrows). Modified Gram stain, × 340

Fig. 4 High magnification micrograph of a collection of *N. [illegible]* spores. Polar vacuoles are indicated by arrows. Modified Gram stain, × 2040



Fig. 5 Greatly thickened lepto-meninges, with massive extravasation of red cells which, in great parts, obscures other changes. A thrombosed artery is indicated by an arrow. Typical cerebral cellular *Nosema* lesion in the lower right of the picture. H&E, × 50.

Microscopic Findings

a. Light Microscopy

Vascular alterations. In the acute stages of involvement, the affected arterial segments showed degeneration, extending to fibrinoid necrosis, the lesions frequently being eccentric but, affected at times also the entire circumference of the vessels (Fig. 2). Endothelial damage was often seen, sometimes with desquamation of endothelial cells. Destruction of internal elastic membrane and thrombosis were frequent events in fresh lesions. The damaged vessel walls were rapidly infiltrated with polymorphonuclear cells and eosinophils. Collections of *Nosema* organisms were very common findings within endothe

lial cells, or in medial smooth muscular elements (Figs. 3–4) but it was also obvious that the parasites frequently occurred extracellularly following rupture of parasitized cells. In somewhat older lesions the infiltrating inflammatory cells consisted predominantly of mononuclear cells, frequently of the plasma cell type. In later developmental stages the injured vascular tissue and thrombosed arterial segments were replaced by fibro-cellular elements resulting in nodular thickenings of the arterial walls, scarring and luminal narrowing or sometimes complete obliteration (Figs. 5–6). At this stage of involvement parasites were seldom seen.

Accumulations of inflammatory cells occurred perivascularly sometimes with formation of granulomatous tissue, either diffusely distributed, or as individual granulomas, closely connected with adventitial tissue.

Distribution of arterial lesions. The arterial lesions were found in a variety of organs and tissues examined. The coronary arteries were affected in nearly all cases. Of 108 animals which all displayed coronary arterial alterations, 49 foxes also had hepatic arterial lesions, and 23 corresponding changes in the kidneys. A high incidence of vasculitis affecting medium sized and minor arteries was also observed in striated muscles, peripheral nerves, spleen, lungs, gonads, stomach and intestine. In the central nervous system there was, as a rule, widespread vasculitis, also involving the veins.

Extra-vascular lesions. Extra-vascular lesions were most advanced in the kidneys and the central nervous system. Renal lesions consisted of extensive interstitial nephritis, the infiltrating inflammatory cells being mostly of the plasma cell type. These cells were frequently pyroninophilic. Myriads of parasites were, in acute cases, present within tubular epithelial cells and in tubular lumina. Cerebral lesions consisted of severe meningo-encephalitis with microgranulomas; collections of parasites were often seen, in and outside these lesions. Changes at other sites included focal granulomatous lesions in the liver and heart muscles. Groups of parasites



Fig. 6. Obliterative renal arterial lesions and interstitial nephritis. H&E, × 100

were, in many cases, seen elsewhere than the kidneys and the brain, including inside epithelial cells in excretory ducts of salivary glands. Hyperplastic processes with proliferation of pyroninophilic cells were frequently noted in lymph nodes, spleen and bone marrow in older cases.

A number of eyes has been studied. Characteristic arterial lesions were also found in the eyes; the result of this study will be published in a separate report (2).

b. Electron Microscopy

Electron microscopy was performed in order to identify the parasites. Groups of spores, at various stages of maturation, were easily found in cerebral vessel walls. They were often present extra-cellularly and were



Fig 7 Electron micrograph of group of *Nosema* spores within the wall of a cerebral artery Some sections of polar filaments are indicated by arrows. × 30,000.

Fig 8 Electron micrograph of a mature *Nosema* spore in the wall of a cerebral artery oblique and cross sections of a characteristic polar filament. × 60,000.

easy to identify when the characteristic polar filaments were discernible (Figs. 7–8)

DISCUSSION

Our observations demonstrate that arterial alterations, similar to classical polyarteritis nodosa, are very common complicating lesions in fox nosematosis. Although incipient arterial modifications have been noted previously in a cat (20) and in dogs (17) in association with nosematosis, there are, as far as we are aware no earlier reports of fully developed polyarteritis nodosa in other species connected with this disease. The most common lesions, associated with nosematosis in all mammalian species, are focal granulomatous encephalitis, and interstitial nephritis (3 13 17 18) Thus, the extra-vascular changes observed in our material correspond closely to those occurring in other species.

The aetiology and pathogenesis of polyarteritis nodosa are incompletely understood A common view is, however that this vascular damage reflects immunological disturbances, probably of the hypersensitivity type (7) In domestic animals, polyarteritis nodosa is known to occur in association with both

certain bacterial infections (19) and viral infections (6) this vascular malady has also been thought to occur in connection with another protozoan parasite, *Sarcocystis* in calves (5)

Whether the arterial lesions reported in this paper depend on the presence of parasites within the damaged vascular walls, or on an altered immunological state remains in doubt. Foxes affected with nosematosis exhibit a pronounced hypergammaglobulinaemia (12) an observation which supports the view that the vasculitis in our material has an immunological background. Further immunological studies are necessary to clarify this point.

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HETEROGENEITY IN *IN VITRO* RESPONSE TO PROGESTERONE AND MELPHALAN OF MAMMARY TUMOURS INDUCED IN THE RAT BY 7, 12 DMBA

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Aspegren, K. Björklund, A. & Tropé, C. Heterogeneity in *in vitro* response to progesterone and melphalan of mammary tumours induced in the rat by 7 12 DMBA. Acta path. microbiol scand. Sect. A, *84* 297–300 1976.

Five mammary tumours induced in rats by 7 1 DMBA were each divided into four sections, and studied as cell suspensions *in vitro* for response to melphalan or progesterone measured as H^3-thymidine incorporation. Heterogeneity towards melphalan and progesterone was found within all five tumours, but the mode of reaction of the two drugs differed. The biological relevance of these findings is discussed.

Key words Mammary tumours, rat, induced progesterone and melphalan response *in vitro*

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In a previous work (1) effects of steroid hormones on cell suspensions from rat mammary tumours and sarcomas were demonstrated *in vitro*. Inhibitions as well as stimulations were found in the two types of tumour. It was thus suspected that steroid response *in vitro* could be an unspecific toxic effect. However different steroids had different effects on one and the same tumour (1) suggesting a "specific" rather than a general toxic effect.

As shown by *Håkansson & Tropé* (5) methylcholanthrene-induced mouse sarcomas are sensitive to cytostatic drugs *in vitro*. Moreover there is correlation between effects *in vitro* and *in vivo* in the transplantation situation (6). It has also been shown that clones with different sensitivity to cytostatic drugs are present within a single tumour (4).

Steroid hormones are frequently used in the treatment of human mammary carcinoma, but irrespective of the initial success, this treatment will often fail. The mammary tumour induced in the rat by 7 12 DMBA is also a hormone-sensitive tumour which often has been used in experimental work as a model of tumour in man. It was therefore considered of interest to study the heterogeneity of this tumour with regard to the *in vitro* response to a steroid hormone. In order to investigate whether the arresting effect of the hormone was similar to that of a cytostatic drug the suspensions were simultaneously tested with melphalan.

MATERIAL AND METHODS

Mammary tumours were induced in 50-day-old Sprague Dawley female rats (Anticimex, Stockholm) by intragastric application of 20 mg 7 12

certain bacterial infections (19) and viral infections (6) this vascular malady has also been thought to occur in connection with another protozoan parasite *Sarcocystis* in calves (5)

Whether the arterial lesions reported in this paper depend on the presence of parasites within the damaged vascular walls, or on an altered immunological state remains in doubt. Foxes affected with nosematosis ex hibit a pronounced hypergammaglobulin aemia (12) an observation which supports the view that the vasculitis in our material has an immunological background Further immunological studies are necessary to clarify this point.

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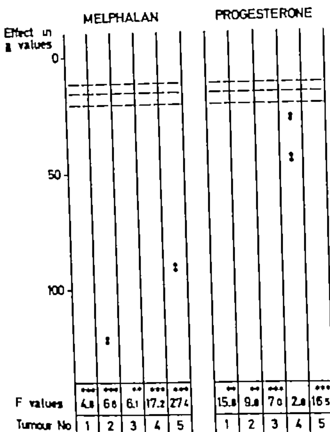


Fig 1 Effect of melphalan and progesterone on rat 7 12-DMBA-induced mammary tumours. Each tumour was divided in four parts which were simultaneously and separately tested with the two drugs. Significance of effects (difference in incorporation of H^3-TdR into control and drug tested tubes) is indicated by dotted lines at the top of the graph. Variance ratios (F-values) and significance levels, estimating the variance between quarters of a tumour are given at the bottom. NS = not significant * = $0.05 > p > 0.01$ ** = $0.01 > p > 0.001$; *** = $p < 0.001$

mary tumours and sarcomas in the rat (virus or chemically induced) the latter type of tumour not generally being considered hormone responsive (1) However different steroid hormones in similar concentrations have different *in vitro* effects on cell suspensions from rat mammary tumours (1) The lack of correlation between effects of melphalan and progesterone in the present investigation shows that sensitivity to steroid hormones in an *in vitro* system is not like the sensitivity to an alkylating agent such as melphalan. Thus, unspecific features such as rate of proliferation, tumour cell permeability etc are unlikely explanations of the variable response to added drugs. The observed *in vitro* toxicity of steroid hormones seems to be a "specific toxicity" characteristic of certain cell populations rather than a "general toxicity" as postulated by *Nordqvist* (5) The "specific toxicity" concept is further strengthened by the fact that a vividly proliferating normal rat tissue remains unaffected of high concentrations of steroid hormones *in vitro* (2) Thus it is reasonable to assume that *in vitro* tests of the effects of steroid hormones reflect phenomena of biological relevance although they do not necessarily reflect hormone sensitivity or unsensitivity *in vivo* of tumours.

Heterogeneity of response of tumour quarters was found to apply to melphalan as well as to progesterone in all tumours although the significance of the heterogeneity in response to progesterone was weak and probably incidental in one tumour In another tumour there was no effect of progesterone in two pieces while two other pieces from the same tumour responded. This could indicate a variability in hormone sensitivity among different cell clones within one and the same tumour If this is true of human mammary

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carcinoma as well, it is easy to understand why the percentage of failures of initially successful hormone treatment is high. If the present finding of cell clones with variable sensitivity to cytostatic drugs and steroid hormones is applicable to the human situation, the prospects of reliable *in vitro* cell culture methods by which to predict response to cytostatic drug or steroid hormone are not very bright simply because of the difficulty involved in obtaining representative material from a given tumour.

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Acta path. microbiol. scand. Sect. A, 84 301–305 1976

PRIMARY CARCINOMA OF THE SEMINAL VESICLE

Case Report

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Kindblom, L.-G & Pettersson, G Primary carcinoma of the seminal vesicle. Case report. Acta path. microbiol. scand. Sect. A, 84 301–305 1976.

A 72-year-old man with a seminal vesicle carcinoma is reported. The patient was treated by local excision of the tumour and radiotherapy When the patient deteriorated and radiological and scintigraphic signs of skeletal metastases developed, hormone therapy (oestrogen) was initiated. Within a few weeks the patient was free from his severe pain. The radiological and scintigraphic signs of metastases had either diminished in size or disappeared after one year of oestrogen therapy The patient is still alive and well 2 years after the diagnosis was established.

Key words: Adenocarcinoma oestrogen therapy seminal vesicle

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Primary carcinoma of the seminal vesicle is exceedingly rare. By a review of the literature from 1871 to 1946, *Lazarus* collected 20 cases of carcinoma which according to the reports originated in the seminal vesicles. In 13 of these cases, neoplastic involvement of the prostate gland had also been demonstrated. Since it proved difficult to exclude the possibility of a secondary involvement of the seminal vesicle from a primary carcinoma of the prostate, he listed only 7 cases as authentic primary carcinomas of the seminal vesicle. In a critical review of the literature in 1956, *Dalgaard & Giertsen* accepted 23 cases as primary carcinomas of the seminal vesicle. Since then, occasional cases have been reported (*Marshall et al.* 1961 *Ewell* 1963 *Lansberg & Iaroshinskij* 1964 *Rodrigues Rees* 1964 *Kaplan* 1965 *Dawson & Meltz* 1965 *Balint* 1965 *Smith et al.* 1966, *Hajdu & Faruque* 1968, *Saidnejad* 1970 *Lipets* 1970) The tumours, with only a few exceptions (*Saidinejad* 1970) were encountered in elderly men. In more than half of the patients metastases developed and the mortality rate was high (*Lazarus* 1946, *Dalgaard & Giertsen* 1956)

In the present paper a case of adenocarcinoma originating in the seminal vesicles of a 72 year-old man treated by oestrogenic hormone and kept under observation for 2 years is described.

CASE REPORT

A 72-year-old man, previously in good health apart from repair of herniae in 1927 and 1940 and a pelvic fracture in 1966, was admitted to hospital in November 1973 on account of acute urinary retention. Emergency catheterization released 1450

nd of urine. During the preceding 2 years, the patient had experienced difficulty in micturition. Rectal examination revealed a massive prostatic enlargement which was thought to be due to benign nodular hyperplasia. The rectal mucosa was smooth and freely mobile. Pre-operative intravenous urography showed a dislocation of the distal parts of both ureters but no obstruction. The patient was operated upon in November 1973 at the Surgical Department of Mölndal Hospital, Sweden. Transvesical enucleation of prostatic adenomas produced 50 g of typical adenomatous tissue. Rectal examination was performed to verify the completeness of the enucleation whereupon a large mass could still be palpated. Via an incision through the prostatic capsule and the trigonum, 50 g of grey brown, gritty tissue was excised by blunt dissection from the retrovesical space. The postoperative course was uneventful and the patient was discharged 14 days after the operation, free from urinary complications. Since the histological examination revealed an adenocarcinoma considered to originate from the seminal vesicles, the patient received 6000 rad in the prostatic region.

In April 1974 the patient was re-admitted to hospital with increasing back pain. Scintography of the skeleton showed multiple osteolytic metastases to the ribs, vertebrae and pelvis. Radiological examination, as compared with earlier radiograms, revealed an increased compression of vertebra L_1, the skeletal structure of which was irregular and reduced density of vertebra L_2. On chest X-ray elevation of the right cupola of the diaphragm and a slight right-sided pleural effusion were observed. Laboratory tests showed a haemoglobin concentration of 13.3 g/100 ml, a sedimentation rate of 65 mm in 1 hour and an alkaline phosphatase activity of 16 Buch and Buch units (upper limit 8). Further radiotherapy 3600 rad was directed towards the site of the pain.

In June 1974 the patient was again totally disabled by severe pain which was relieved by dextropropoxyphen and pethidine. His general condition was deteriorating rapidly. At this stage, hormone therapy was initiated. Polyoestradiol phosphate (Estradurin) 160 mg/month for the first three months and then 80 mg/month intramuscularly along with ethinyl oestradiol tablets (Etivex) 50 mg twice daily for 14 days and subsequently 50 mg daily were prescribed.

Four weeks after the institution of this therapy the patient was free from pain and able to resume his normal daily activities.

At follow-up examination in August 1975, 21 months after the operation, the patient was still free from pain, had gained in weight and was in excellent condition. Scintography of the skeleton showed marked regression of the osteolytic areas. Radiological examination showed sclerosis of the osteolytic vertebrae L_1 and L_2. No new metastases were in evidence. The haemoglobin concentration was 11.7 g/100 ml, the sedimentation rate was 28 mm in 1 hour and the alkaline phosphatase activity was reduced to 2 Buch and Buch units.

PATHOLOGY

Histological Methods

The surgical specimen was fixed in 4 per cent formaldehyde solution and embedded in paraffin. Five micron thick sections were stained according to the haematoxylin-van Gieson method and with haematoxylin and eosin. The PAS-reaction (McManus), Sudan black and long Ziehl-Neelsen were used to identify lipofuscin.

Gross Appearance

The retrovesical tumour tissue weighed 50 g and appeared in several pieces, the largest of which measured 3 cm in diameter. The tumour tissue was friable and its cut surfaces were greyish or grey-brown and of gritty appearance. The prostatic tissue weighed 50 g and was seen as 2 separate nodules with grey-white or pale pink cut surfaces; they were of a tough, fibrous consistency.

Microscopic Appearance

The histological pattern of the retrovesical tumour varied considerably, for the most part appearing as a rather poorly differentiated adenocarcinoma, in some areas almost solid (Fig. 1) but frequently of an adenomatous structure with prominent papillary projections (Fig. 2). The tumour cells were medium-sized with distinct cytoplasmic borders and a vesicular nucleus with one or two prominent nucleoli. Within these poorly differentiated parts of the tumour were areas composed of irregular glandular or cribriform structures lined by a pseudostratified epithelium of rather clear polygonal cells (Fig. 3 and 4). These areas possessed some of the characteristics of the normal seminal vesicle. However, no distinct lipochrome pigment granules were demonstrated within the

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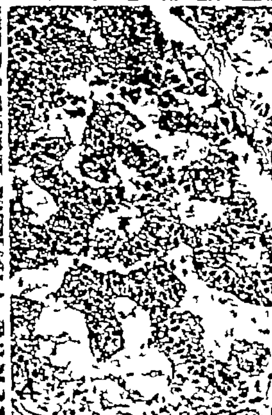
Fig. 1. Solid low differentiated area of seminal vesicle adenocarcinoma. H&E × 30.

Fig. 2. Prominent papillary and adenomatous structures. H&E × 90.

Fig. 3. An area of the tumour showing irregular cribriform structures lined by polymorphic cylindric pseudostratified epithelium. H&E × 180.

Fig. 4. Seminal vesicle-like area within the tumour. H&E × 120.



epithelium neither in these areas nor in the poorly differentiated areas. Scant fibrus stroma containing many fairly wide capillary-like vessels and occasional, small groups of lymphocytes lay interspersed between the tumour cells.

The prostatic tissue contained hyperplastic glands, some of which were cystic, lined by a single row of columnar epithelial cells without atypia. A slight lymphocytic infiltration was observed within the hyperplastic fibromuscular stroma. Careful examination of the prostatic tissue did not reveal any signs of malignancy

DISCUSSION

Sedondary neoplastic involvement of the seminal vesicles is not infrequent. Carcinoma of the prostate shows a predilection for the seminal vesicles which may also be involved in an infiltrating rectal carcinoma (*Dalgaard* & *Giertsen* 1956, *Mostofi* & *Price* 1973) Metastatic rectal shelf tumours are frequently located around the vesicles (*McCrea* 1948, *Dalgaard* & *Giertsen* 1956) Thus, the differential diagnosis of an adenocarcinoma involving the seminal vesicles may be difficult. In the present case the appearance of a nodular hyperplastic prostate and a large retrovesical tumour at surgical exploration together with the absence of malignant changes within the microscopically examined prostate favours the diagnosis of a primary seminal vesicle carcinoma. Furthermore the histological picture of the tumour presenting features like those of a papillary adenocarcinoma with seminal vesicle-like areas very well conforms to the histological features of seminal vesicle carcinomas previously described (*Dalgaard* & *Giertsen* 1956, *Dawson* & *Mekie* 1965 *Hajdu* & *Faruque* 1968) The clinical course of the disease contradicts the possibility of a malignancy elsewhere with metastatic spread to the seminal vesicles.

It is well-known that growth of the seminal vesicles in rats is stimulated by testosterone administration and profoundly depressed by orchidectomy Bearing this in mind, it is surprising that we have found only 3 reports concerning 3 patients where attention has been drawn to the possible benefit of hormone therapy by oestogenic administration and orchidectomy (*Marshall et al.* 1961 *Rodriguez Kees* 1964 *Smith et al.* 1966) In the present case the clinical response to oestrogen administration was dramatic within a few weeks of initiating the hormone therapy the patient was free from his severe pain and his general condition was improving It is of interest that the radiological and scintographic signs of metastases had either diminished in size or disappeared after one year of oestrogen therapy These observations indicate the definite value of hormone therapy in the management of seminal vesicle carcinoma.

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LIPOSARCOMA OF THE MENINGES

A Case Report

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Sima, A. Kindblom, L.-G. & Pellettieri, L. Liposarcoma of the meninges. A case report. Acta path. microbiol. scand. Sect. A, 84 306–310 1976.

A report on a liposarcoma in the meninges of a 70-year-old woman is presented. Pre-operative clinical and angiographic findings indicated that the tumour might be a meningeoma. Pathological examination revealed a predominantly lipoma-like well-differentiated liposarcoma with round-cell and pleomorphic areas. The angiographic and pathological differential diagnosis are discussed.

Key words: Liposarcoma; meninges.

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Intracranial malignant tumours arising in the meninges and their derivates are rare According to *Kernohan & Uihlein* (1962) sarcomas account for 3.0 per cent of primary intracranial tumours, including reticulum cell sarcomas, haemangiopericytomas and "giant cell fibrosarcomas" The majority of intra cranial sarcomas has been classified as fibrosarcomas, spindle-cell sarcomas, and poly morphic-cell sarcomas (*Christensen & Lara* 1953) Chondrosarcomas as well as chordomas originating within the skull base may ex tend intracranially (*Heffelfinger et al* 1973) Occasional cases of chondrosarcomas and un specified myxoid soft tissue sarcomas have also been reported to arise intracranially (*Kernohan & Uihlein* 1962)

Liposarcomas are probably the most common soft tissue sarcomas showing a predilection for the extremities and the retroperitoneum (*Enzinger & Winslow* 1962 *Ackerman & Rosai* 1974 *Kindblom et al.* 1975) To our knowledge liposarcomas have not previously been reported to arise from the central nervous system, although *Stout & Lattes* (1967) have mentioned that sporadic cases of liposarcomas may occur in the meninges. In a Swedish national series of 122 liposarcomas diagnosed between 1958–1966, no intracranial liposarcoma was encountered (*Kindblom et al.* 1975)

The present paper reports a 70-year-old woman with a primary liposarcoma in the meninges which, on the basis of the clinical and angiographic findings, was considered to be a meningeoma.

CASE REPORT

Clinical course A 70-year-old woman, previously healthy suddenly became ill in February 1975 and sought medical care because of disturbances of speech, dizziness and difficulties in gait. After a couple of weeks she seemed to improve but then again deteriorated, presenting additional loss of memory stepping gait and dysphasia. The patient,



Fig. 1. External carotid angiogram showing the blood supply to the tumour from a. meningea media.



Fig. 2. Internal carotid angiogram illustrating the blood supply from a. pericallosa and branches from a. cerebri media.

examined by a general practitioner was supposed to be suffering from Parkinson's disease in an early stage and was medically treated though without effect.

In June 1975 when the patient became somnolent and developed *expressive aphasia* she was admitted to a county hospital. On admission it was noted that the patient also had right-sided hemiplegia and urinary incontinence. Echo-encephalography showed a non-significant dislocation of the mid-line to the right (3.5 mm). Electro-encephalography showed spasmodic and paroxysmal abnormalities over the left hemisphere, distortion being maximal in the frontal and frontotemporal divisions. Lumbar puncture revealed a liquor protein content of 98 mg per cent. The patient's symptoms were then *interpreted as sequelae of* cerebrovascular accident, and the patient received medical care as well as physical and aphasic training. To exclude the possibility of a tumour a left-sided carotid angiogram was obtained in July 1975 which revealed a 6 × 6 × 5 cm large "meningeoma" on the convexity of the left hemisphere (see below). The patient was referred to neurosurgical treatment. The results obtained by pre-operative routine-laboratory examinations were within normal limits except for an elevated SR, 55 mm/hour. At operation, a well delineated tumour was found to be attached to the dura and to the pia. The tumour was located close to the superior sagittal sinus, but not penetrating the sinus itself. It was radically removed.

After the operation the patient improved: she gained consciousness but a *right-sided hemiplegia* persisted. The patient was discharged to return to the country hospital for further care. Three months after the operation, the patient was completely alert, able to walk by herself and the strength in the right side improved to almost normal.

Angiographic findings. The blood supply to the lateral parts of the tumour was derived from the medial meningeal artery (see Fig. 1) while the medial parts of the tumour were supplied from the pericallosal artery and other branches of the anterior cerebral artery (see Fig. 2). A homogeneous opacification of the tumour was observed both at external and internal angiography. The pericallosal artery was dislocated 5 mm to the right and the internal cerebral vein 10 mm to the right.

PATHOLOGY

Gross appearance. The operative specimen consisted of a 6 × 6 × 5 cm large lobulated, well *delineated tumour (Fig. 3).* A 4 × 4 cm large piece of the dura was attached to the tumour. At



Fig. 3. Macroscopic appearance of the biopsy specimen. To the right, thin rim of brain parenchyma. *At the bottom, pieces of attached dura can be seen.*



Fig. 4 Liposarcoma infiltrating the dura (top A H&E, × 30) and compressing the underlying brain tissue (bottom B H&E, × 30). Wide vessels are numerous within the tumour and the adjacent dura (A, B). The tumour reveals areas with numerous multivacuolated atypical lipoblasts with hyperchromatic scalloped nuclei (C, H&E, × 186) and highly cellular pleomorphic areas (D H&E, × 120)



Fig. 5 Numerous wide blood-filled vessels within the tumour (A, H&E, × 30) most of the irregular vessels being capillary-like (B, H&E, × 120)

one side of the tumour a thin rim of brain parenchyma was seen. T the gross view the tumour was sharply defined from the brain tissue. The cut surfaces of the tumour were of a pale, brown-yellow colour and of a consistence softer than that to be expected of a meningeoma.

Histological methods The specimen was fixed in 10 per cent formalin and embedded in paraffin. Five μ thick sections were routinely stained according to the haematoxylin-van Gieson method and with haematoxylin-eosin. Weigert's lastin-haematoxylin-van Gieson method was used for the demonstration of elastic tissue of essels. The PAS-method (McManus) was performed with and without prior digestion with diastase. Oil Red O on frozen sections was used for the demonstration of fat.

Microscopic appearance The tumour was closely attached to and partly infiltrating the dura (Fig. 4 A) extending into the subdural space and compressing the underlying brain substance (Fig. 4 B). Histologically most area of the tumour were lipomatous, showing conspicuous, large acuolated cells, univacuolated cells of ariable size and several multivacuolated cells with hyperchromatic large scalloped nuclei, in other words, feat res of atypical lipoblasts (Fig. 4 C). Some areas differed by their higher cellularity showing either closely attached rounded or ovoid cells with hyperchromatic nuclei and only occasional, cytoplasmic vacuoles (Fig. 4 D) or large polymorphic multivacuolated lipoblasts. Scattered mitotic figures were seen in these cellular areas. On frozen sections, the uni- and multivacuolated atypical fat cells were shown to contain Oil Red O positive material. In areas of degeneration, the cytoplasm of some cells re ealed large hyaline PAS-positive globules. The tumour was highly vascular with conspicuous, large, irregular angulated vessels of capillary-like type (Fig. 5 A). The essels were lined by flat, endothelium-lik cells surrounded by varying amounts of fibrous tissue (Fig. 5 B). There were no elastic fibres or muscle tissue components within the walls of the vessels. The dural essels close to the tumour, both of venous and arterial type were wide and appeared dilated.

DISCUSSION

According to WHO (1969) liposarcomas have been classified into 5 subtypes well

differentiated, myxoid, round-cell pleomorphic and mixed type. A subdividing of the mixed types into predominantly well differentiated types and predominantly myxoid types with round-cell and/or pleomorphic areas has been suggested and proved to be of prognostic significance (*Kindblom et al.* 1975). The present tumour was of the predominantly well-differentiated type with pleomorphic and round-cell areas. According to the pre-operative investigation, the clinical features, the angiographic appearance and the localization of the tumour favoured a meningeoma. Because of the exceedingly rich vascularity of the tumour seen at angiography a meningeoma of "angioblastic type" or "richly vascular type" was considered most likely.

Histologically there should be no problems in distinguishing between meningeoma and liposarcoma. It is interesting that xanthomatous areas with groups of foam cells containing Oil Red O positive cytoplasmic material may occur in angioblastic meningeoma (*Rubinstein* 1972). However the angioblastic meningeomas also show areas typical of meningeoma and they do not reveal any multivacuolated lipoblasts. In richly vascular meningeomas, the vessels are more thick walled and hyalinized (*Rubinstein* 1972) than in liposarcomas (*Kindblom et al* 1975).

The possibility of a metastasis must be considered whenever a liposarcoma is found at an unusual site. In the present case there were no clues to suggest a primary liposarcoma elsewhere.

It is noticeable that also a hibernoma has been described to arise in the meninges (*Vagn Hansen* & *Osgård* 1972). Hibernoma is a benign highly vascular tumour angiographically showing irregular vessels and signs of rapid circulation; the angiographic findings are usually held to be contributary signs of malignancy (*Angervall et al.* 1964; *Kindblom et al.* 1974). The angiographic appearance of 24 liposarcomas involving the extremities has been studied (*Kindblom et al.* 1975). A correlation between type of liposarcoma and vascularity was found; the round-cell liposarcomas and pleomorphic liposarcomas were most vascular frequently showing signs of rapid circulation. It is interesting that the present tumour appeared to be even more vascular than the extremity liposarcomas of pleomorphic and round-cell types. This difference may be due to a richer vascular supply at the site of origin.

We are indebted to the *Roentgendiagnostic Department*, Rungdh Hospital for permission to reproduce the angiograms.

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THYMIC CARCINOID

A Case Report

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Sundström, C. & Wilander E. Thymic carcinoid. A case report. Acta path. microbiol. scand. Sect. A, 84 311–316, 1976.

A case of thymic carcinoid appearing in a 56-year-old man is presented. No endocrine disturb-ances are apparent. Mediastinal lymph node metastases were found at operation. The tumour was positive with Grimelius argyrophil silver stain. Ultrastructurally neurosecretory granules were seen.

Key words: Thymic carcinoid carcinoid, thymic.

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There is strong evidence to indicate that the group of thymic tumours comprises a subgroup with a histogenesis different from that of the rest. Since *Macadam & Vetters* (10) first described the presence of neuro-secretory granules in some thymic tumours, this observation has been made by a few other authors (7 11 14 16, 20) These granules are not found in all thymic tumours (6 21) These tumours exhibiting granules ultrastructurally have been described histo-logically as carcinoid-like or as "epidermoid thymoma (10) and "carcinoma of the thymus (9) Carcinoid-like thymic tumours, like many foregut carcinoids, have shown a positive argyrophilic reaction. Some of these tumours have been associated with endocrine disturbances. Most of them have been asymp-tomatic but in some cases there has been a concomitant multiple endocrine adenomatosis (11 17) The group described as epidermoid thymoma has regularly been associated with Cushing's syndrome (12, 18 22) ACTH activity has been observed in tumour extracts in a few of these cases (7 12 14)

The following report describes a male pa tient without endocrine disturbances who was operated on for a mediastinal mass. The tumour had a definite carcinoid histology but contained areas of cellular pleomorphism an unusual feature in these tumours. Ultra structurally neurosecretory granules were de monstrated.

CASE REPORT

Clinical Dat

The patient was a 56-year-old concrete worker He had had rheumatic fever at the age of 15 44 years old cholecystectomy was performed. Since the age of 47 he had suffered from angina pectoris with pain on effort and exposure to coldness. Four months prior to the present admis-sion to hospital, diabetes mellitus was detected.

The patient was admitted to hospital after a period of precordial pains of increasing severity Myocardial infarction was suspected, but ECG and GOT GPT and LDH were normal. On chest X ray a mediastinal expanding lesion was seen. An aortic aneurysm was suspected, but aortography

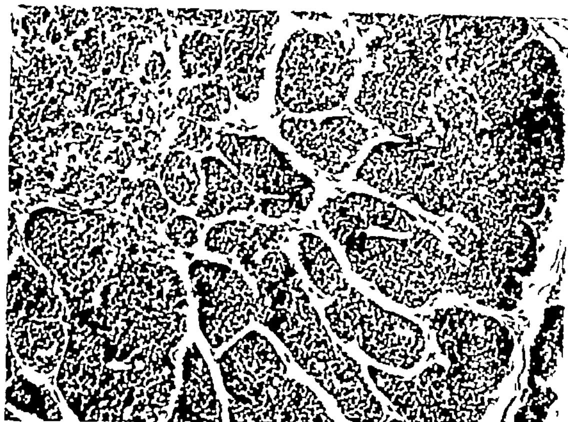


Fig. 1 Overview of thymic carcinoid, showing the border between the two parts of the tumour. To the right the part with regular nests of epithelial cells separated by thin fibrous septa. To the left the part of the tumour exhibiting irregular nests of tumour cells with cellular pleomorphism. (H-E, × 50)

showed normal conditions. Simultaneous coronary angiography revealed pronounced narrowing of the left coronary arterial branches. The right coronary artery was occluded. The patient had no symptoms of an endocrinologic nature, apart from his diabetes mellitus.

The patient was transferred to the department of thoracic surgery. On admission no symptoms of cardiac insufficiency were noted. A systolic murmur grade I–II was heard over the apex. Blood pressure was 150/80. At f ne needle aspiration biopsy of the mediastinal mass a diagnosis of possible thymoma or malignant lymphoma was made. At operation, via median sternotomy the tumour was found in the upper anterior mediastinum within the thymus. It was macroscopically encapsulated and seemed to be demarcated from the normal thymic tissue. It was of the size of a grapefruit, of hard consistency and adherent to the pericardium. The tumour together with a rim of the thymus and the adherent part of the pericardium was resected. Two enlarged lymph nodes were found in the upper mediast um. As the findings in the frozen sections from the tumour nd lymph nodes indicated a malignant thymoma, a coronary by-pass operation was not performed.

The patient recovered w ll from the operation. Radiotherapy was given in two sessions (total dose 6300 rads). During the following eight months the attacks of angina pectoris became more frequent and attacks of dyspnoea also occurred. Eleven months after removal of the thymic tumour the severity of the dyspnoea increased beyond therapy and the patient died, presenting a picture of cardiac failure.

Autopsy

Autopsy revealed cardiac hypertrophy (heart weight 725 g) involving both ventricles. In the posterior wall of the left entricle several fibrous scars were noted. Severe arteriosclerotic obturation was seen in both coronary arteries. The pleural cavities contained 2500 ml of exudate. The lungs were grossly oedematous. Fibrosis was noted in both lungs, especially in the left superior lobe. Fibrosis was also present within the mediastinum. A fragment of the thymus remained. No tumour

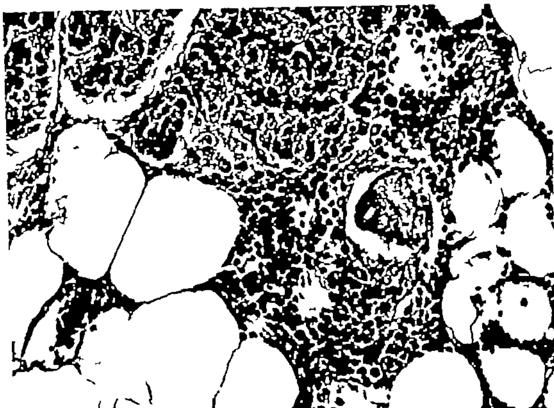


Fig. 2. Detail showing thymic tissue (below) with invading tumour tissue. (H E, × 150)

remnants or metastases were seen in the mediastinum. Neither was any tumour found within the bronchial tree which was thoroughly examined. The gastro-intestinal tract was also carefully examined, but no tumour was found within the oesophagus, stomach, intestines, pancreas or liver. Death was believed to have followed from pulmonary oedema due to cardiac insufficiency and earlier myocardial infarctions.

Light Microscopy

Tumour specimens were fixed in 10 per cent formalin, embedded in paraffin, and sectioned into about 4 μ thick sections which were stained with van Gieson stain, haematoxylin-eosin, the Masson stain (4) and the silver staining methods of *Hellerström & Hellman* (5) and *Grimelius* (3)

The tumour was rich in relatively small homogeneous cells which were accumulated in solid nests of varying size. These nests were separated by a stroma which was arranged in fibrous trabeculae. In one part of the tumour the tumour cells had round or oval nuclei and a moderate amount of cytoplasm. The chromatin was mostly dense and homogeneous. A few cells had small nucleoli. In other parts of the tumour cellular pleomorphism was clearly evident. The nuclei were irregular with vesicular chromatin and nucleoli. Mitotic figures were noted. The fibrous trabeculae in those parts of the tumour were irregular and the cellular nest could not always be defined. In the fat tissue resected together with the tumour involuted thymic tissue was identified. Tumour tissue was also present within this fat and thymic tissue (Fig. 1–2)

The overall picture of the tumour was carcinoid-like, though in some areas the cellular pleomorphism was evident. Using the Grimelius silver stain, moderate amounts of silver granules were seen in the cytoplasm of occasional tumour cells (Fig. 3) The Masson and Hellman-Hellerström stains gave negative results.

Electron Microscopy

For electron microscopic analysis small pieces of the tumour fixed in formalin were post-fixed in 1 per cent osmium tetroxide, dehydrated, and embedded in Epon 812. Sections were prepared with an LKB ultrotome stained with uranyl acetate and lead citrate and studied in a Zeiss EM 9 electron microscope at 60 kV

At the ultrastructural level, a varying number of

granules were seen in the cytoplasm of the tumour cells. The granules were round with a central, moderately electron dense core surrounded by a limiting membrane in which there was a narrow clear space. The diameter of the granules was about 150 μ (Fig. 4) Other intracytoplasmic structures were not sufficiently well preserved to allow detailed ultrastructural analysis.

DISCUSSION

To the author's knowledge, 23 cases of thymic carcinoid have been reported previously (8, 10 11 15 16 17 20) The majority of these tumours have been observed in males of ages ranging between 21 and 72 years. Two females with this type of tumour have been reported (16) Seventeen of the tumours were not associated with endocrine disturbances. In five cases, all males, multiple endocrine adenomatosis was present concomitantly (9 11 17) One case (8) was associated with symptoms of a paraneoplastic nature (neuropathy myopathy polyarthropathy) The malignancy of these tumours has varied. Local invasion and recurrence were noted in four of the cases without endocrine disturbances (15 16) In one of these cases, lymph node metastases were found (13) In a fifth case (8) multiple distant metastases were present. Three of the thymic carcinoids associated with multiple endocrine adenomatosis were clearly malignant, with extensive local invasion or distant metastases, suggesting a more aggressive tumour in this group (17)

The features of the tumour described in this report conforms well in most respects with those generally observed in this type of tumour The thymic origin of this tumour was never doubted The location in the upper anterior mediastinum in close proximity to the thymus itself and with no connection to the main bronchi excluded any other origin than the mediastinum. The histopathology of the tumour was, however unlike that of any other type of thymic tumour (19) Unlike what is normally observed in thymic carcinoids mediastinal lymph node metastases and cellular pleomorphism on histopathological examination was observed in the case presented.



Fig 3 Detail showing a positive argyrophil reaction in scattered tumour cells (Grimelius silver stain, × 500)

The value of ultrastructural and histochemical examination of a thymic tumour with unusual histological features is emphasized. The presence of neurosecretory granules and a positive argyrophilic reaction to the Grimelius silver strain, as found in all gastro-intestinal and in many pulmonary carcinoids (23) strongly suggests the diagnosis of a thymic carcinoid. Whether these findings indicate a release of secretory products by the tumour remains to be determined. In cases of epithelial thymic tumours associated with Cushing's syndrome this is true. In thymic carcinoids the secreted product may be defective and have no clinical effect. This would explain the absence of endocrine disturbances in these cases.

It has been proposed on the basis of light microscopy the common pattern of endocrine disturbances, and the presence of neurosecretory granules in the tumour cells that oat cell pulmonary carcinoma and bronchial

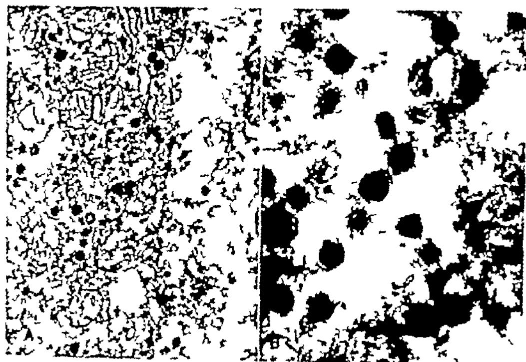


Fig. 4 Electron microphotographs showing neurosecretory granules in tumour cells. a) Tumour cell with several granules in the cytoplasm. (× 70,000) b) Detail of granules with their dense core and surrounding membrane with an interposed clear zone. (× 80 000)

carcinoid tumours are closely related. They may be the malignant and locally malignant tumour respectively derived from Kult schitzky cells which may normally be found in the bronchial tree (1). The same may be true of the type of carcinoma of the thymus associated with Cushing's syndrome and carcinoids within the thymus. There is much to be said in favour of the theory that these two groups of thymic tumours in reality are closely related, viz. the presence of neurosecretory granules, the histopathological picture of an epithelial tumour with an endocrine appearance and the occurrence of endocrine disturbances. These tumours may be derived from neurosecretory Kultschitzky cells present within the thymus. Such cells have been demonstrated in animal thymuses and also in the human thymus in a few cases (16).

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THYMOMA IN A CASE OF SCLERODERMA

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Sundström, C. Thymoma in a case scleroderma. Acta path. microbiol. scand. Sect. A, 84 317 321 1976.

A case of scleroderma with manifestations in the oesophagus and both lungs and a concomitant thymic lympho-epithelioma is presented. This case represents a further documentation of the well-established association between thymoma and collagen disorders.

Key words: Thymoma thymic lympho-epithelioma scleroderma.

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Thymic tumours of different types associated with various diseases have been seen (for review see *Goldstein & Mackay* 1969) the most important of which is myasthenia gravis. The association of thymic tumour and different collagen disorders is also well-established, mostly systemic lupus erythematosus (8, 11 16) and polymyositis (4 10 14 19) Scleroderma has been noted in a case of thymic tumour (12) without further details and in a case of "hyperplasia" of the thymus (2) In a few reports, oesophageal disorders in patients with a thymic tumour have been described dysphagia (3 9) megaoesophagus (5) a case of leiomyoma of the oesophagus (6) Any reports on pulmonary disorders in patients with thymic tumours have apparently not been published.

The following case report gives a description of a patient with a benign thymic tumour together with oesophageal stenosis and pulmonary fibrosis due to scleroderma. This association has never before been described in detail.

CASE REPORT

Clinical data. A 69-year-old woman sought medical help because of dysphagia which had persisted for many years. One year before admission, spots on both lungs were detected by chest X-ray and were interpreted as pulmonary fibrosis. A small tumour was also seen in the hilus of the left lung (Fig 1) Lung function tests revealed a reduced lung capacity of the restrictive type and a reduction of PO at maximal effort. During the last few months prior to admission her dysphagia had aggravated and she had only been able to take liquid food. She had lost in weight, from 58 to 45 kg. At admission, routine laboratory tests gave essentially normal results. Serum electrophoresis showed a polyclonally raised gammaglobulin fraction. X-ray of oesophagus revealed a rather irregular 15 mm long stricture a few cm below the entrance (Fig.) Owing to the stricture the oesophagoscope could not be introduced and dilatation was performed. Any signs of tumour were not observed. After the oesophageal dilatation the patient improved and was sent home, but death occurred shortly after she had left the hospital.

Autopsy findings. Autopsy was performed four days after death. The coronary arteries showed moderate arteriosclerotic changes (weight of heart 270 g) In the anterior mediastinum, a tumour the size of a golf ball was found. Macroscopy failed to reveal a normal thymus. The tumour was well-defined with a relatively thick fibrous capsule. No



Fig 1 Chest X-ray showing a mass on the border between mediastinum and left lung hilus and pulmonary fibrosis.

Fig 2 Oesophageal X-ray showing stenosis (arrow) and prestenotic widening.

metastases were found. The lungs were slightly oedematous (right lung 475 g, left lung 385 g) Mainly in the right lung, but also in the left, the parenchyma was of a fibrous, leathery appearance The wall of the oesophagus was stiff and thickened but otherwise it was of normal appearance. The cortex of the left kidney was moderately reduced (combined weight of kidneys 205 g) Several myomas were seen in the uterus. It was presumed that death had been due to the lung changes.

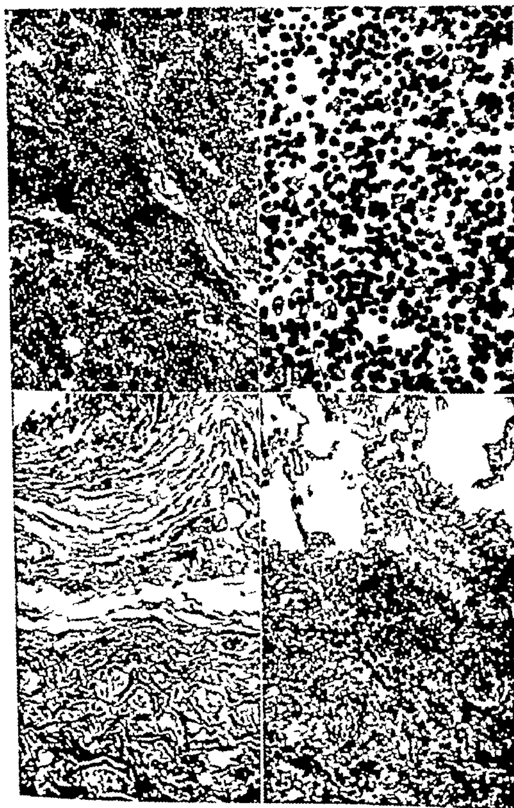
Microscopic examination. The mediastinal mass was partly surrounded by a thick fibrous capsule and septa from the latter extended through the tumour (Fig 3) At external areas of the tumour there was a gradual transition into normal thymic tissue in some places The tumour tissue was composed of small lymphocytes with an intermingled discrete reticulum of epithelial cells. No Hassall's corpuscles of lymphoid follicles were found. A few cystic spaces were seen. Capsular infiltration by lymphocytes wa present in a few places Any cytological signs of mal gn ncy or mitotic figures were not in evidence. The d agnosis was consistent with a thymic lympho-ep thelioma of the lymphocytic type.

The epithelium m he oesophagus was thinned and atrophic. Submuc al precipitates of a hyaline-like partially PAS po substance together with lymphocyte infiltratio were observed Staining reaction for amyloid w negative Plexus myentericus was of normal appearance in the examined parts of the oesophagus. Extensive fibrosis was present in the oesophageal muscles where single or small groups of muscle fibres were separated by strands of fibrous tissue (Fig. 4) Fibrosis in both lungs was extensive. In some areas, the alveolar septa were thickened due to fibrosis. In other areas, fibrosis was confluent and here alveolar structures were compressed or completely absent (Fig. 5) Lymphocytic infiltrates were seen throughout the lungs which were oedematous. No granulomas were seen. Pulmonary small-sized arteries showed scattered intimal lesions with narrowing of the lumen

Fig 3 Microphotographs of the thymic lymphoepithelioma. a) The tumour contained mainly small lymphocytes and thick fibrous septa. (H-E, × 100) b) The lymphocytes were intermingled with epithelial cells. (H E, × 400)

Fig 4 Microphotographs from the oesophagus, showing mucosal atrophy infiltrations of lymphocytes and submucosal fibrosis. (van Gieson, × 100)

Fig 5 Microphotographs from the lung, showing extensive fibrosis, lymphocytic infiltrations, thickening of alveolar septa, and emphysematous widening of alveoli. (van Gieson, × 400)



The changes in the oesophagus and lungs were consistent with visceral manifestations of scleroderma.

DISCUSSION

This patient suffered from two diseases. The manifestations of one of these i.e. the oesophageal stenosis and the pulmonary fibrosis, were diagnosed clinically though scleroderma was never suspected to be responsible. The second disease a thymic lympho-epithelioma was diagnosed at autopsy.

Involvement of the skin and subcutaneous tissues in scleroderma is by no means obligatory. *Tumulty* (18) has pointed out that visceral scleroderma probably precedes the cutaneous form much more frequently than hitherto presumed. Involvement of the gastrointestinal tract is not uncommon and, most often the oesophagus is affected. Our patient suffered from dysphagia due to stenosis of the oesophagus. Involvement of the lungs is fairly common with pulmonary fibrosis and/or pulmonary vascular obstruction (20) and vascular lesions (13). In our patient, pulmonary fibrosis was observed on chest X-rays and at postmortem histopathological examination. Lung function test indicated an impairment of the visco-elastic properties of the lungs and ventilation-perfusion disturbances. These findings are in full accord with *Sackner's* observations in studies of the pulmonary function in cases of scleroderma (15).

The changes observed in the oesophagus and lungs were interpreted as manifestations of scleroderma. Other diagnoses were taken into consideration intake of corrosive chemicals, tumour growth in or near the oesophagus, massive X ray irradiation could be ruled out. The possibility of an acute diffuse interstitial fibrosis (Hamman-Rich) in the lungs seemed unlikely considering the protracted course of the disease and the fact that respiratory distress by no means was prominent.

That the mediastinal tumour was of thymic origin was evident from the location of the tumour its histopathological picture, and from the microscopically verified presence of normal thymus outside the tumour. The microscopic appearance of the tumour is characteristic of the usually benign thymic tumours called lympho-epitheliomas. Capsular infiltrations are common in these tumours, though invasion into adjacent organs is rare (17).

Lattes (12) reported, without detailed description, one case in which scleroderma was associated with a thymic tumour of the granulomatous type. The thymic tumour in his patient was apparently of another type than that described in the present paper. Thus, any detailed reports on cases of combined scleroderma and thymic lympho-epithelioma have apparently not been published before.

The aetiology of these two disorders is unknown. Like systemic lupus erythematosus, scleroderma is associated with an alteration in immune reactivity. A variety of abnormal antibodies are present in the serum (1). Whether this "auto-immunity" plays a primary or a secondary role in the aetiology of the disease, is not known. The aetiology of thymic tumours is entirely unknown. The presence of a tumour in the thymus indicates, however that an immunological disturbance may be involved in some way.

A feature common of the two above described diseases entities might thus be an immune defect. In our case, this possibility was not investigated clinically except for immune-electrophoresis which revealed clearly that titres of IgG IgM and IgA were elevated.

The association between thymic lympho-epithelioma and collagen disorders is well-established. The case presented here offers further documentation in this respect and serves to widen to a certain degree the spectrum of such associations.

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CARCINOID TUMOURS

Frequency in a Defined Population During a 12 Year-Period

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Berge, T & Linell, F Carcinoid tumours. Frequency in a defined population during a 12-year period. Acta path. microbiol. scand. Sect. A, 84 322 330 1976.

The frequency of carcinoids was studied in a 12-year-period (1958–1969) in Malmö, a town with a population of 220,000 at the beginning of the period and 250,000 at the end of it. Of all persons who had died in Malmö, 46 per cent were necropsied in the first year of the study and 70 per cent in the last (altogether 62.6 per cent) The series was examined in a uniform way at *one* department of pathology Carcinoid tumours were found in 1.22 per cent (199 patients) of patients comprised in the entire necropsy series (16,294 autopsies) Bronchial carcinoid accounted for 0.1 per cent, the remaining lesions were found in the digestive tract. About 90 per cent of the carcinoids were found incidentally at necropsy During the same period 44 carcinoids were diagnosed in surgical specimens examined in Malmö. The average annual frequency of carcinoid in the entire series was about 8.4 per 100,000 inhabitants, which is about 7 times as high as that recorded in the National Cancer Register applying to the whole of Sweden The value of such country-wide reports is discussed. The carcinoid syndrome is extremely rare and was observed only once during the entire 12-year-period. The localization, frequency of metastases and sex-distribution of carcinoids are described and discussed in detail.

Key words: Carcinoid tumours; frequency in defined population.

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The frequency of carcinoids varies notably from one series to another This is, at least partly due to differences in composition of the series and in the extent of the post mortem examinations. It was therefore considered justified to publish the results obtained in an analysis of findings obtained in a series studied throughout 12 years, examined by a uniform method and composed of a fairly well defined population. Part of the study has been published earlier (*Linell & Månsson* 1966)

MATERIAL AND METHODS

The series was collected in Malmö, a town in the south of Sweden. At the beginning of the 12-year period in 1958 the town had 220,000 inhabitants and, by the end of 1969 it had increased to 250,000 It is not possible in this context to give a complete survey of the population for each year Part of it has already been described (*Berge* 1967) and complete description is planned (*Berge & Lundberg*) An attempt has been made, however to estimate the type and degree of selection of patients in the necropsy series (*Berge* 1967)

It is only in exceptional cases that residents of the community die in other hospitals. Non-residents are excluded from our necropsy series

Malmö has only one general hospital, one hospital for chronic diseases, and one mental hospital. The university institute of pathology Malmö Gen-

eral Hospital, serves all the hospitals in the city About 99 per cent of all persons who die in hospitals are necropsied Of all those who died in Malmö in the beginning of the 12 year-period, 46 per cent were examined post mortem. The corresponding figure applying t the end of the period was as high as 70 per cent In total, 62.6 per cent were necropsied. The study covered the 12-year period, 1958–1969 During that period of time, 16,294 necropsies were performed (Table 1 Fig. 1)

Necropsy was performed in a uniform way throughout the investigation. The gastro-intestinal canal was slit up rinsed and examined macroscopically Gross changes were examined microscopically At all necropsies, tissue for microscopic examination was taken from all lobes of the lungs, the myocardium, kidneys, liver and spleen In patients with malignant tumours, the microscopic examination was extensive and included not only the primary tumour but also metastases, if any and regional, mediastinal, para-aortic and supraclavicular lymph nodes. During certain periods, also the axillary and inguinal lymph nodes were examined routinely

Carcinoids diagnosed in surgical specimens during the period in question were also included. Carcinoids were diagnosed mainly on a morphological basis. All cases were not examined routinely for argentaffinity and/or argyrophilia. The results of such examinations of material obtained at necropsy are not always reliable.

RESULTS

In the necropsy series, altogether 8,214 malignant neoplasms were diagnosed, 201 (2.4 per cent) of which were carcinoids. The frequency and number of carcinoids relative to the total number of primary tumours in individual organs varied widely *viz.* 2.4 per cent (18 out of 747) in the lung, 0.8 per cent (5 out of 651) in the stomach, 95 per cent (152 out of 160) in the small intestine, 36.8 per cent (7 out of 19) in the appendix, 1.8 per cent (13 out of 706) in the colon, 1 per cent (3 out of 314) in the rectum and 0 4 per cent (1 out of 271) in the liver

The 201 carcinoids were demonstrated in 199 individuals, in other words a frequency of 1.22 per cent in the necropsy series. In 2 cases, carcinoids were found at 2 different sites. One patient had one carcinoid of the ileum and one of the appendix In the other one carcinoid involved the ileum and one colon No metastases were found in either case. Multiple carcinoids at *a single* site were regarded as a single case.

The vast majority of the carcinoids were

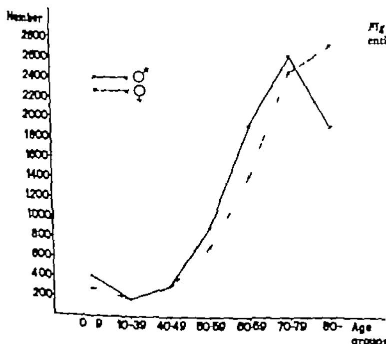


Fig 1 Age distribution in the entire necropsy material.

TABLE 1 *Age and Sex Distribution of Inhabitants Who*

		Age groups (years)			
		0–9	10–19	20–29	30–39
Male	D	473	102	210	325
	N	401 (84.8 %)	28 (27.5 %)	44 (21.0 %)	102 (31.4 %)
Female	D	321	54	108	1 6
	N	275 (85.7 %)	23 (42.6 %)	4 (43.5 %)	104 (59.1 %)
Total	D	794	156	318	501
	N	676 (85.1 %)	51 (32.7 %)	91 (28.6 %)	206 (41.1 %)

TABLE 2. *Carcinoids in Necropsy Series*

Site of primary tumour	All tumours			Metastasizing tumours			Not known before necropsy	Clinically known or suspected malignancy primary tumour not located
	f	m	tot	f	m	tot		
Unknown	1	1	2	1	1	2	1	1
Bronchus	16	2	18	1	2	3	14	1
Stomach	0	5	5	0	4	4	2	1
Small intest.	54	98	152	18	25	43	134	7
Appendix	4	3	7	0	0	0	7	0
Large intest.	7	6	13	4	2	6	7	2
Rectum	1	2	3	0	0	0	3	0
Liver	1	0	1	1	0	1	1	0
Total	84	117	201	25	34	59	169	12
							90.0 %	

TABLE 3 *Sites of Carcinoids of the Small Intestine in the Necropsy Series*

	Solitary	Multiple	Total
Duodenum	5		5
"Small intestine	11	3	14
Ileum + jejunum	0	3	3
Jejunum	8	2	10
Ileum	78	40	118
Meckel' diverticulum	2	0	2
	102	50	152

situated in the gastro-intestinal tract. The bronchial carcinoids constituted barely one tenth of the total number

The distribution of the carcinoids is given in Table 2. The table also includes the number of metastasizing tumours, tumours that

...ed (D) and of Those Necropsied (N) in 1958–1969

Age groups (years)				
40–49	50–59	60–69	70–79	80–
710 313 (44 1 %)	1700 905 (53.2 %)	3036 1928 (63.5 %)	4034 2605 (64.6 %)	3140 1921 (61.2 %)
468 312 (66.7 %)	980 701 (71.5 %)	1984 1417 (71 4 %)	3658 2465 (67 4 %)	4569 2703 (59.2 %)
1178 625 (53.1 %)	2680 1606 (59.9 %)	5020 3345 (66.6 %)	7692 5070 (65.9 %)	7709 4624 (60.0 %)

had escaped detection *ante mortem* and tumours that had been known or suspected *ante mortem* but not been located. In only 20 (10 per cent) of the cases, the tumour had been correctly diagnosed. Since these patients died during the period covered by the investigation they were included in the necropsy series and not in the biopsy series.

The sites of solitary and multiple carcinoids of the small intestine are given in Table 3

The age and sex distribution of the carcinoids of various parts of the body are given in Table 4

A carcinoid was demonstrated only in one individual below 40 years, namely in a 15-year-old girl with tuberous sclerosis who had died from bronchopneumonia

Necropsy revealed one carcinoid of the appendix which had not been known *ante mortem*.

Metastases were found in 29 4 per cent of the cases. The number and frequency of metastases to various organs from different primary tumours are given in Table 5 The frequencies are given in relation to the total number of metastasizing tumours at the different sites.

Eighty-one (40 7 per cent) out of the 199 patients with carcinoids had one or more co-existing malignant tumours of different histological type.

Biopsy material. Also this material was obtained during the 12-year period, 1958–1969 The series comprised 44 individuals. The sites of the tumours are given in Table 6

The age and sex distribution is shown in Table 7

Six (13.6 per cent) of the individuals in the biopsy series had a co-existing malignant tumour of different histological type. In ad-

TABLE 4 *Age Distribution at Time of Diagnosis Necropsy Series*

Age group	Gastro-intestinal f	Gastro-intestinal m	Bronchial f	Bronchial m	Other f	Other m	Total f	Total m
10–19	1	0	0	0	0	0	1	0
40–49	1	4	0	0	1	0		4
50–59	4	6	1	0	0	0	5	6
60 69	16	35	2	1	0	0	18	36
70–79	17	38	6	0	0	1	23	39
80	28	31	7	1	0	0	35	32
Total	67	114	16	2	1	1	84	117

TABLE 5 *Number of Metastases. (Frequencies for Carcinoids of the Small Intestine Resp. Total Number)*

Site of metastases	Site of primary tumour Unknown No.	Bronchus No.	Stomach No.	Small intest. No. %	Large intest. No.	Liver No.	Total No. %
Lung	2	1	2	1 (2.3)	2		8 (13.6)
Pleura			1				1 (1.7)
Small intestine					1		1 (1.7)
Colon				1 (2.3)			1 (1.7)
Liver	2	1	4	14 (32.6)	5		26 (44.1)
Pancreas	1		1	2 (4.7)			4 (6.8)
Peritoneum				6 (14.0)	1	1	8 (13.6)
Myocardium				1 (2.3)			1 (1.7)
Skeleton	1		1		1		3 (5.1)
Spleen				2 (4.7)			2 (3.4)
Lymph nodes	2	1	3	42 (97.7)	4	1	53 (89.8)
Thyroid			1	1 (2.3)			2 (3.4)
Adrenal	1				1		2 (3.4)
Kidney	1		1				2 (3.4)
Ovary				1 (2.3)		1	2 (3.4)

TABLE 6. *Carcinoids in Biopsy Series*

Site of primary tumour	All tumours f	m	tot	Metastasizing tumours f	m	tot
Bronchus	5	0	5	0	0	0
Stomach	0	2	2	0	1	1
Small intestine	3	6	9	1	3	4
Appendix	19	3	22	0	0	0
Large intestine	0	2	2	0	1	1
Rectum	1	3	4	0	0	0
Total	28	16	44	1	5	6

TABLE 7 *Age Distribution in Biopsy Series*

Age group	Gastro-intestinal f	m	Bronchial f	m	Total f	m
10–19	4	1	0	0	4	1
20–29	3	0	0	0	3	0
30–39	3	2	0	0	3	2
40–49	6	2	2	0	8	2
50–59	4	5	2	0	6	5
60–69	2	4	0	0	2	4
70–79	1	2	1	0	2	2
Total	23	16	5	0	28	16

dition, one patient had undergone conization because of *in situ* carcinoma of the portio.

All the carcinoids of the small intestine were localized to the ileum. Seven patients had only one tumour while one had 2 and one had 6.

The carcinoid syndrome had been reliably diagnosed only in one patient, a 74-year-old woman in whom the urinary HIAA-values varied between 25 and 50 mg/100 ml/24 hours. Necropsy revealed extensive carcinoid growth in the peritoneum and skeleton. But no metastases to the liver were demonstrable. Metastases were detected in 6 (15.6 per cent) of the patients in the biopsy series.

DISCUSSION

The frequency of gastro-intestinal carcinoids varies widely in the necropsy series on record. Several examiners have found frequencies of about 0.5 per cent, while frequencies ranging from 0.08 per cent to 1.36 per cent have been found by others (for ref. see *Linell & Månsson* 1966). The frequencies may vary in different parts of the world, but the highest frequency given is probably the one closest to the true frequency because it was found in a special prospective investigation of the incidence of intestinal tumours (*Feyrter* 1934, 1962).

The discrepancies between the anatomic localization and age distribution of the tumours in biopsy series and in necropsy series have been pointed out earlier (*Foreman* 1952). These discrepancies may probably be ascribed to

1. early obstruction of the lumen of the appendix and inflammation of the latter indicating appendectomy,
2. the high frequency of appendectomy as compared with resections of other parts of the gastro-intestinal tract may result in the discovery of asymptomatic tumours of the appendix, and
3. relatively detailed examination of biopsy specimens from the appendix, less detailed examination of specimens obtained at necropsy.

As many of 90 per cent of all carcinoids demonstrated at necropsy had not been diagnosed *ante mortem*. Most of the tumours had been clinically insignificant and were discovered at necropsy of patients who had died from some other disease. As expected, the age distribution was therefore the same as that in the entire necropsy series.

Many of the carcinoids in the biopsy series had also been discovered incidentally. Thus, 4 of the 5 bronchial carcinoids had been diagnosed accidentally at roentgen examination. As regards the carcinoids of the small intestine, 2 had been discovered by histological examination of Meckel's diverticulum which was the site of a perforating ulcer while the others were diagnosed at examination indicated by ileus or suspected appendicitis, or at operation indicated by some other neoplasm.

Ten of the 22 carcinoids of the appendix were found in appendices removed *en passant* at gynaecological operations. In the other 12 cases, the appendix had been removed because of diagnosed or suspected appendicitis. It was only in 3 cases that the surgeon observed gross changes compatible with a diagnosis of carcinoid. In surgical specimens obtained in the present study the frequency of carcinoids of the appendix is too low because not all appendices removed during certain periods were sent to us for pathological examination.

The 4 rectal carcinoids had been discovered at proctoscopy.

The fact that so many carcinoids are incidental findings at necropsy or by routine histological examination of surgical specimens must imply that they as a rule are slow growing tumours and that most of them metastasize late or not at all. In other words, most of the patients die *with* their carcinoid(s) and not because of it.

Carcinoids are diagnosed in all age groups. The youngest patient described was only 10 days old (*Forbes* 1925) and, as already

mentioned, in our series the age distribution was the same as that in the entire necropsy series. Accordingly the total number of individuals with carcinoid(s) is probably rather large but unfortunately it cannot be reliably calculated on the basis of findings in the present or in other series.

About one third of the carcinoids of the small intestine were multiple carcinoids (Table 2) The strong tendency of these tumours to be multiple has been described by among others, *Kuiper et al* (1970) who in their series of 30 cases found that 20 per cent were multiple carcinoids and by *Moertel et al.* (1961) who found a frequency of 29 per cent in their series of 209 cases.

Moertel et al (1961) held that there might be some relation between carcinoids and other malignant tumours, but the postulation is hardly correct. Since carcinoids grow slowly time permits other malignant neoplasms to develop at practically the same frequency in these patients as in the population in general.

Malignant tumours were demonstrable in 44.5 per cent of the individuals comprised in the necropsy series. The frequency was almost the same viz., 40 7 per cent, in the patients with carcinoids. Such high frequencies have also been reported by *Warren & Coyle* (1951) according to whom the frequency ranged at 53 per cent and *Foreman* (195?) according to whom it ranged at 47 per cent. In a recent investigation by *Kuiper et al.* (1970) the frequency of a co-existing malignant tumour was 31.9 per cent. This frequency would certainly have been higher if the post mortem examinations had been more extensive.

Moertel et al. (1961) found that 47.3 per cent of the co-existing tumours involved the gastro-intestinal tract. In the series reported by *Kuiper et al* (1970) the frequency was 43.5 per cent a[illegible] compared with 35 8 per cent in our series. This corresponds well to the ratio between t[illegible] total number of tumours of the digestive t[illegible] nd all the 8,213 tumours thus, it does n[illegible] gest that the alimentary tract is the site [illegible] redilection of co-existing tumours. In 12 [illegible] nts, the co-existing tumour was situated in the lung. Ten of these tumours were squamous epithelial carcinomas, one was an adenocarcinoma and only one was a small-celled bronchial carcinoma.

According to *Ritchie* (1956) figures denoting the frequency of metastases range between 21 per cent and 75 per cent. In the present series it was 29 4 per cent. The frequency of metastases must, of course vary inversely with the necropsy frequency since thorough necropsy including routine examination of the intestine will reveal many small tumours without metastases.

In conformity with findings in a recently published large series of carcinoids (*Hajdu et al* 1974) lymph nodes and the liver were the most common sites of metastases. Of all 59 metastasizing carcinoids, 53 (89.8 per cent) had set up secondaries in lymph nodes. (This was the case in 97 7 per cent of the metastasizing carcinoids of the small intestine.) Secondaries were found in the liver in 44 1 per cent (26 out of 59) The distribution of carcinoid metastases over various parts of the body does not differ notably from that of other epithelial tumours. Metastases were more frequent in cases of multiple carcinoids of the small intestine than in cases of solitary tumours. Twenty-nine (58 per cent) of the former but only 14 (13 7 per cent) of the latter had metastasized.

In the biopsy series (13.6 per cent) the frequency of metastases was only about half as high as that in the necropsy series. That the frequency in the former series was lower is no matter for surprise since many of these tumours had been excised—*en passant*—at an earlier stage in younger individuals.

MacDonald (1956) found peptic ulcer in 38 per cent of the patients with metastasizing carcinoid, compared with 5.5 per cent in those without. *Kuiper et al.* (1970) did not find any increased frequency of ulcers in his patients with carcinoids.

Ulcer was found in 28 (14 1 per cent) of our 199 necropsied patients with carcinoid. This frequency does not differ (significantly) from the 17.9 per cent found in the necropsy series studied in 1969 In the latter series, the

ratio of gastric ulcer to duodenal ulcer was 2 1 In patients with carcinoids, this ratio was 8 1 We can offer no explanation of this difference.

With a view to evaluating the annual frequency of carcinoids in patients in Malmö all cases diagnosed before 1958 were excluded. There were, however only 3 such cases (2 carcinoids involving the small intestine and 1 involving the colon) Since the annual frequency of tumours as rare as carcinoids may vary widely from year to year we decided to calculate the average frequency throughout the period. During the 12 year period the population in Malmö increased from 220,000 up to 250 000 The 242 carcinoids diagnosed during that period corresponded to an annual frequency of 8 4 per 100,000 (as regards females, the frequency was 7 4 per 100 000 and among males 9 4 per 100,000) A minor part of this study has been published earlier (*Linell & Månsson* 1966) At that time, the frequency was stated to be about 1 per 100 000 per year but the calculation was based only on findings in biopsy specimens which gives an incomplete picture of the frequency of these tumours. Nevertheless, it is noteworthy that the other findings in the smaller series agree well with those obtained in the large series, thereby suggesting that the examination method was uniform throughout the period.

As previously mentioned our figures are lower than the true frequency A comparison with figures recorded in national cancer registers should be of interest. In most of these registers, however it is not possible to detect the frequency of carcinoids since they are grouped together with other tumours, but in the Swedish register carcinoids are tabulated separately (Cancer incidence in Sweden 1959–1965) In this 7-year period, the annual frequency was about 1.2 per 100 000 *i.e.* the frequency found by us was 7 times as high. This is but natural because the Cancer register comprises cases from all parts of Sweden and the average of necropsies performed is low

Age differences cannot explain the difference in frequency As already mentioned, all patients with carcinoid(s) except one were ≥40 years of age. In the Swedish population 1959–1965 43 6 per cent of the males and 46 0 per cent of the females belonged in these age groups. The corresponding frequencies in the Malmö population were 42.2 per cent and 46.8 per cent, respectively As our figures certainly are minimum figures, the value of whole-country-reports is questionable when frequencies in different countries or places are to be compared.

Carcinoids of the digestive tract are generally considered to be more common in men than in women. This was confirmed in the present study and is mainly due to the fact that carcinoid of the small intestine was twice as common in men as in women. In the Swedish Cancer Register the ratio was about 1 1

As regards carcinoids of the appendix, however the ratio is inversed, such carcinoids being about 3 times as common among women as among men. This marked difference is due entirely to the fact that females predominate in the biopsy series. The explanation of this preponderance may be that the appendix is removed *en passant* during gynaecological operations and subjected to histological examination. In our series, carcinoids were diagnosed in this way in 10 cases, but the surgeon had observed gross changes only in three of these. *Foreman* (1952) reported that 84 per cent of the carcinoids involving the appendix were seen in women, and he referred to larger series reported in the literature where the frequencies ranged from 65 to 82 per cent. We are in agreement with his explanation of the predominance of females.

Among children, however females seem also to predominate in series of patients with appendicitis. In a series of 30 children younger than 15 years, 29 presented with signs or symptoms of acute appendicitis. Twenty three of the patients were girls and there was histological evidence of acute appendicitis in 25 of the 30 cases (*Rydén et al.* 1975)

Kulpert et al. (1970) reported that 8 out of 14 carcinoids of the appendix in patients in their biopsy series were asymptomatic. *Shorb & McCune* (1964) declared that 10 out of 25 carcinoids of the appendix were discovered incidentally. In *Foreman's* (1952) series, the corresponding figures were 10 out of 18.

We cannot explain the striking predominance of women in the series of bronchial carcinoids where the ratio was 8:1. Such sex difference has not been observed in any other series. *Kreyberg's* (1969) large series of 71 cases included 45 males and 26 females. *Salyer et al.* (1975) found bronchial carcinoids in 15 men and 13 women in a series of 28 patients.

Carcinoid of Meckel's diverticulum is relatively rare, but according to *Sanders & Axtell* (1964) 30 cases are on record. 17 of these had metastasized. Carcinoids involving Meckel's diverticulum were found in 2 cases in the necropsy series and in 2 in the biopsy series. No metastases were found in any of these 4 cases. The 2 individuals in the biopsy series had been operated upon on account of a perforating ulcer in an ectopic gastric mucosa. The 2 cases in the necropsy series were incidental findings.

In 2 cases of metastasizing carcinoid it was not possible to locate the primary tumour.

The carcinoid syndrome was diagnosed only once (0.5 per cent). This complication must therefore be considered quite unusual (lit. see *Ringertz*, 1967).

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THYMOMA METASTASIZING TO EXTRATHORACIC SITES

A Case Report

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Nickels, J & Franssila, K. Thymoma metastasizing to extrathoracic sites. A case report. Acta path. microbiol. scand. Sect. A, 84 331–334 1976.

In the case presented, metastases to extrathoracic sites developed in a 53-year-old man four years after the diagnosis of inoperable thymoma had been established. The appearance of metastases was preceded by an extensive intrathoracic invasion of the tumour The thymoma was of epithelial subtype which was the most common (67 per cent) type also in the previously published 29 cases of thymomas metastasizing to extrathoracic sites.

Key words Thymoma, metastasizing case report.

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Thymomas metastasizing to extrathoracic sites are rare Using strict criteria, we found only 29 cases in the literature (1–7 9–18, 20–22) We should like to add one case which was included in an earlier published series of thymomas (19) in which case metastases to extrathoracic sites developed later We have also reviewed the previously published cases in order to find whether histological or other features might suggest that they deviate from other thymomas.

CASE REPORT

In the autumn of 1969 a building worker born 1921 felt chest pains. He had previously been healthy except for a pyelonephritis 10 years earlier. The chest roentgenogram was normal, but later on, in July 1970, the mediastinum was seen to be dilated. Bronchoscopy revealed an extrathoracic process which compressed the trachea anteriorly above the bifurcation. The muscular strength was normal without signs of myasthenia gravis. At operation, the mediastinum was found to be the site of a tumour that was adherent to the sternum and growing around the aorta and the great arteries and veins. Radical operation was impossible and only a biopsy was taken. The tumour was found to be a thymoma, predominantly of epithelial type according to *Lattes'* (1962) classification. Radiation therapy (betatron-foton) was given using a tumour dose of 4900 rads throughout 7 weeks. *Late in 1972*, right-sided pleural exudate was found to contain cytologically malignant cells. Cytostatic treatment (cyclophosphamide-vincristine) was started Late in 1973 sudden paralysis of the lower extremities developed. Myelography showed an extradural expansion including spinal compression at the level of the thoracic vertebrae IX–XI. Radiation therapy by a tumour dose of 3000 rads to the area was given but the symptoms were not relieved. In August 1974 metastases to the seventh rib to the right were visualized by roentgenological examination, and palpable tumours were found in both axillae. Biopsy showed a metastasizing tumour a thymoma predominantly of epithelial type. Cytostatic treatment (cyclophosphamide-vincristine) was started again. The mediastinal tumour was roentgenologically seen to compress the main bronchi of the right middle and lower lobes. At

the terminal stage heavy oedema of the right arm and both lower extremities developed The patient died in March 1975

Autopsy Findings

The whole mediastinum was filled with the tumour which invaded the right lung and compressed the right bronchial tree The tumour invaded also the visceral and parietal pleura as well as the thoracic wall and grew through the vertebral discs of the thoracal vertebrae XI–XII to the epidural space, compressing the spinal cord. The parietal and the visceral pericardium were totally invaded, but the myocardium was not involved. The tumour was seen to extend directly through the diaphragm, around the right adrenal gland and the hepatic vein and into the liver (Fig 1) The tumour penetrated the hepatic vein from the liver growing intraluminally in the inferior vena cava to the right atrium. The intestinal venous plexus showed a high degree of stasis.

Separate metastases without connection with the primary mediastinal tumour were seen in the lower lobe of the left lung (diameter 1.5 cm) in lymph nodes of both axillae (diameter 0.5–2 cm) right iliac area (diameter 2 cm) and lesser curvature of the stomach (diameter 2 cm)

Histological Findings

Histological sections of the primary biopsy of the mediastinal tumour revealed tumour tissue which was divided into lobules by prominent fibrous septae (Fig. 2) The tumour cells had a round to oval nucleus which was larger and paler than that of the lymphocytes (Fig 3) The cytoplasm was clear and moderately abundant. The cells were PAS-negative and showed no argentaffine reaction or argyrophilia. Any remarkable variation in the shape or size of the tumour cells was not seen. The mitoses were scanty The tumour was composed solely of these cells of epithelial appearance. No spindle cells were seen and only a few scattered lymphocytes could be found (Fig. 3) Reticulin fibres divided sheets of tumour cells into smaller islands. The histological picture corresponds to that of a thymoma, predominantly of the epithelial cell type (13) In the autopsy specimens, the mediastinal tumour presented the same kind of histological picture No marked pleomorphism was seen. In the areas of direct extension of the tumour and in the extrathoracic metastases, the histological picture was in other respects the same, but the broad collagen bands were lacking (Fig 4) No marked pleomorphism or increased frequency of mitoses was seen

DISCUSSION

In the literature we found reports on 43 cases of thymomas presenting extrathoracic metastases. Using strict criteria, however we could accept only 30 cases (1–7 9–18, 20–22) including the present case

The predominant cell type in metastasized tumours was epithelial in 67 per cent (20 cases) which contrasts with the frequency of 16–45 per cent (mean 27 4 per cent) (1 11 13) of this type in thymomas in general. The difference is statistically significant (χ^2-test, $p<0.001$) In other respects the histology of metastasizing thymomas had not been found to differ from that of thymomas in general. In only two primary thymomas metastasizing to extrathoracic sites (16, 20) the mitotic frequency was elevated. In different series of thymomas, the frequency of extrathoracic metastases appear to be 1 4–15.5 per cent (mean 6.6 per cent) (1 6 11 13 19 20) According to this, the frequency of metastases from tumours, predominantly of epithelial type is about 16 per cent.

In seven cases (4 5 16 18 22) the primary tumour was 10 cm or smaller in diameter at the time of appearance of metastases. Metastases to the liver were most frequently seen (60 per cent) followed by the kidney (24 per cent) and different extrathoracic lymph nodes (24 per cent) Metastases to the brain were found in 3 cases.

The distribution by age of patients with

Fig 1 Tumour growth around the hepatic vein in the liver

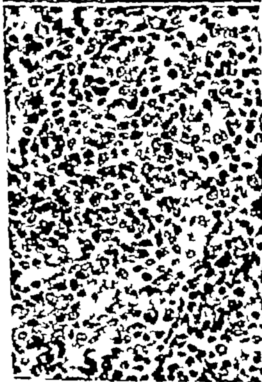
Fig 2 Mediastinal thymoma divided into lobules by prominent fibrous septae. Haematoxylin and eosin. × 55

Fig 3 Predominantly epithelial type of thymoma. Biopsy from mediastinum. The tumour is composed of cells of epithelial appearance. Some scattered lymphocytes can also be seen. Haematoxylin and eosin. × 500.

Fig 4 Lymph node metastasis in the axilla. The broad collagen bands are lacking, but reticulin fibres divide the metastatic cells into smaller groups. Silver impregnation method for reticulin fibres. × 310.


1
0
10
20
30


2





metastasized thymomas did not differ from that of patients with other thymomas, but a male preponderance was seen (1 8 1) The average time of survival after the diagnosis had been established was 3.5 years (17 cases) and after the appearance of extrathoracic metastases 1.5 years (12 cases)

Myasthenia gravis was seen in 30 per cent of the patients with metastasized thymomas, which corresponds to the 30–46 per cent frequency in thymomas in general (1 8) One of the patients in whom metastases developed presented with thrombocytopenia (6) and one with agammaglobulinaemia (7) One had an additional epidermoid carcinoma of the lung (2) and one had breast carcinoma (11)

CONCLUSIONS

Extrathoracic metastases from thymomas in general are rare, but they seem to occur in cases of thymomas, predominantly of epithelial type at a frequency much higher than that in other types. Other features such as the size of the primary tumour or cellular pleomorphism and mitotic frequency seem to be of little help in predicting the possible metastatic behaviour of the thymoma.

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A STUDY OF RUSSELL BODIES IN HUMAN MONOCLONAL PLASMA CELLS BY MEANS OF IMMUNOFLUORESCENCE AND ELECTRON MICROSCOPY

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Blom, J., Mansa, B. & Wiik, A. A study of Russell bodies in human monoclonal plasma cells by means of immunofluorescence and electron microscopy. Acta path. microbiol. scand. Sect. A, 84: 335-349, 1976.

Five patients with a serum M component were shown to possess plasma cells containing Russell bodies. Four of the patients suffered from multiple myeloma, whereas the fifth probably had a different disease or was in a premyeloma stage. The Russell bodies stained blue with the May-Grunwald-Giemsa stain and were found both in the nuclei and in the cytoplasm of the plasma cells. Ultrastructural studies showed that the Russell bodies were osmophilic and those located in the cytoplasm were always situated within the cisternae of the rough endoplasmic reticulum. The intranuclear Russell bodies were always surrounded by a triple layered membrane, and some evidence was obtained that these bodies were first formed within the perinuclear space of the cells. Immunofluorescence studies using anti-L chain conjugates showed a positive marginate staining of the intranuclear as well as the cytoplasmic Russell bodies of the cells from all patients. Only one patient had cells with Russell bodies which also stained positive with an anti-H chain conjugate. All Russell bodies were PAS negative, irrespective of their location in the plasma cells. It is concluded that some plasma cells in multiple myeloma may produce an excessive amount of L chains which, in combination with a failure in the secretion of immunoglobulin molecules, may lead to the formation of Russell bodies.

Key words: Electron microscopy; immunofluorescence; multiple myeloma; plasma cells; Russell bodies.

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Russell bodies (Rb) (1) are cytoplasmic or intranuclear granules which stain moderately to strongly basophilic in conventionally stained smears, e.g. with the May-Grünwald-Giemsa stain (19). By electron microscopy the Rb are seen as dense osmophilic globules the major[ity] of which are homogeneous. They are located within the cisternae of the rough endoplasmic reticulum with a clear space between the body and the external wall. Intranuclear locations of Rb have also been described (19).

Bone marrow aspirates obtained from a series of patients with multiple myeloma were studied by light and electron microscopy and five patients who had plasma cells containing

Russell bodies were found. The Russell bodies have been further characterized by light and immunofluorescence microscopy as well as by electron microscopy and in addition, the M components of the patients sera have been classified.

Our immunofluorescence studies suggest that the Rb irrespective of their location within the cell are composed mainly of free light chains of immunoglobulin.

MATERIAL AND METHODS

Light Microscopy

Smears of bone marrow aspirates were fixed in absolute methanol and stained with May-Grünwald Giemsa (M-G-G) at pH 6.5. The periodic acid Schiff (PAS) reaction was carried out using Harris haematoxylin as counterstain (28). Photomicrographs were taken with a Carl Zeiss photomicroscope II using Agfachrome 50 L professional film.

Electron Microscopy

Bone marrow aspirates were immediately injected into a solution containing 3 per cent glutaraldehyde (TAAB Laboratories, Emmer Green, Reading, England) dissolved in 0.1 M sodium cacodylate buffer pH 7.2 (24) with 0.01 M $CaCl_2$ and fixed in this solution for 1 hour at room temperature (20–23 C). The clumps of clotted cells and marrow fragments were then cut into 1 mm cubes and fixation was continued in fresh fixative of the same composition for another 4 hours at room temperature. After transfer to 0.2 M sucrose in 0.1 M cacodylate buffer pH 7.2 with 0.01 M $CaCl_2$, the specimens were stored overnight at 4 C. They were then postfixed for 1 hour at room temperature in 1 per cent osmium tetroxide in 0.1 M barbiturate buffer pH 7.3 with 4.5 per cent sucrose added (8).

After fixation the specimens were treated en bloc for 1 hour with 2 per cent uranyl acetate in barbiturate buffer without sucrose and dehydrated in increasing concentrations of alcohol followed by propylene oxide treatment (17). The tissue cubes were then transferred to a mixture of equal volumes of Vestopal W (Martin Jaeger Geneva, Switzerland) containing 2 per cent initiator and per cent accelerator and propylene oxide and left overnight in an open vessel at 30 C. During the night the propylene oxide had evaporated and the next day the specimens were transferred to fresh Vestopal. They were allowed to soak in fresh plastic monomers for two periods of 24 hours each prior to final embedding (23). Polymerization was carried out 24 hours at 37 C followed by 48 hours at 60 C.

Ultrathin sections (ca. 50 nm) were cut with glass knives on an LKB ultrotome III microtome collected on Formvar covered carbon reinforced 200 mesh copper grids, and post-stained with magnesium uranyl acetate (9) and lead citrate (22).

Electron microscopy was carried out on a Philips EM 300 electron microscope at 60 kV using primary magnifications of 1500 and 9000 ×. Exposures were made on Kodak Fine Grain Release Positive Film Type 5302 and developed in Kodak D19 b developer. Suitable fields were photographically enlarged as desired, and for this paper about 2500 recordings were studied.

Immunoelectrophoretic analysis was carried out according to *Scheidegger* with some modifications described earlier (16). Polyvalent anti-human serum raised in rabbits was used (16) and the M components were further classified by the use of antisera specific for human α, γ, μ, and δ heavy chains and κ and λ light chains. The antisera to γ, μ, κ and λ were purchased from Dakopatts, Copenhagen, the anti-α and -δ sera from Behringwerke Marburg, and the anti-ε serum from Pharmacia, Uppsala.

The *protein concentration* of the serum samples was determined with an AO refractometer series PR A (American Optical). The concentration of the M components was estimated either by free electrophoresis according to Tiselius or by single radial immunodiffusion (18) on plates containing the appropriate antiserum. The WHO preparation 67/97 was used as standard for IgG and IgA.

Immunofluorescence Studies

The bone marrow specimens were studied by a direct immunofluorescence technique according to the method of *Hijmans et al.* (12). IgG fractions of antisera specific for human α, γ, μ, κ and λ chains were purchased from Dakopatts (Copenhagen). All the samples showed a single specific precipitin line when examined by crossed immunoelectrophoresis according to *Laurell* (15). They were all labelled with tetramethylrhodamine-isothiocyanate (TMRITC) as described by *Cebra & Goldstein* (7). Samples of fluorescein-isothiocyanate (FITC) labelled rabbit IgG specific for human α, γ, μ, κ, and λ chains were purchased from Dakopatts. All conjugates used were shown to be specific by direct immunofluorescence tests in which monoclonal bone marrow cells from patients with multiple myeloma and macroglobulinaemia were used (12).

In all cases double staining experiments were carried out on one and the same slide of a smear in order to determine whether cells containing one particular type of heavy chain also contained only one type of light chain.

Monoclonal cells containing particular heavy

or light chain were counted in each cytocentrifuge preparation and the percentage of cells containing the different chains was calculated (11 14). The κ/λ ratio was always determined from one slide stained with both FITC-anti-κ and TMRITC-anti-λ. For each specimen the percentage of monoclonal cells in the total number of nucleated cells observed by phase contrast microscopy was calculated (14). Finally the percentage of Russell body containing cells in the monoclonal cell population was also calculated.

Case Report 1

Female patient (EJ) born 1928. A serum M component of IgG type lambda in a concentration of 26 g/litre was found in 1965. 6 per cent plasma cells were present in the nucleated cells of the bone marrow and the blood showed an erythrocyte sedimentation rate (ESR) of 82 mm/hour. No Bence Jones (BJ) protein was present in the urine.

The patient was followed until 1971 without any treatment. By then the concentration of the M component had increased to 98 g/litre and plasma cells now amounted to 60 per cent of the nucleated cells. X-ray examination showed no abnormalities of the skeleton. The ESR was increased to 140 mm/hour and BJ protein was now found in the urine.

Treatment was started with melphalan and later continued with cyclophosphamide. In 1973 the patient suffered from several attacks of pneumonia and eventually died in the summer of that year. Autopsy showed bilateral bronchopneumonia, but no special comments on the skeleton were given in the report.

Case Report 2

Female patient (EH) born 1914. In 1973 serum from this patient was found to contain an M component of IgA type kappa and the diagnosis of multiple myeloma was established. Treatment with melphalan was initiated but resulted in a pronounced anaemia which necessitated regular blood transfusions and consequently cyclophosphamide was substituted for melphalan at the end of 1974. No BJ protein was found in the urine. At the time of this study the ESR was 145 mm/hour and the number of plasma cells amounted to 25 per cent of the nucleated cells of the bone marrow.

Because of an increase in the concentration of the serum M component and in the plasma cell number to over 75 per cent of the nucleated cells the treatment was again changed to melphalan instead of cyclophosphamide in May 1975.

Case Report 3

Female patient (NN) born 1918. In 1972 a slightly elevated ESR of 50 mm/hour was found in the blood. No other clinical symptoms could be demonstrated. A serum sample however showed an M component of IgG type lambda.

In 1975 the ESR was 45 mm/hour and except for the M component the results of all other common laboratory tests were within normal limits. X-ray examination of the skeleton, now as in 1972, showed no abnormalities. BJ protein was not present in the urine.

Several examinations of the bone marrow since 1972 showed that the plasma cells amounted to 5–8 per cent of the nucleated cells and with no change in the relative number of plasma cells containing Rb. In M-G-G stained smears the plasma cells all appeared atypical. They were 13–20 μm in diameter and generally their vacuolated cytoplasm stained slightly basophilic.

The patient is still without clinical symptoms and is controlled periodically; no treatment with cytostatics has been given.

Case Report 4

Male patient (OB) born 1932. In 1972 a plasmocytoma in vertebra lumbalis III was diagnosed. The plasmocytoma was treated with radiotherapy in all 5000 R. Since then X-ray examination of the skeleton has shown no sign of dissemination.

In serum and urine an M component of kappa light chains was found. The concentration of BJ protein in the urine was 1.0 g/litre. By free electrophoresis the serum M component showed a mobility corresponding to β globulin, but was not seen as a distinct peak. A broad γ globulin peak accounted for about 3 g/litre. The BJ protein in serum was readily demonstrated within the γ-1 mobility range by immunoelectrophoretic analysis. The concentration of this component was judged to be at least as high as in the urine. The total protein concentration in serum was found to be 64 g/litre. Other laboratory tests showed that the ESR and Hb values, as well as the WBC and platelet counts in the blood, were within normal limits. The plasma cells in the bone marrow varied from 5–11 per cent of the nucleated cells, but the relative number of plasma cells containing Rb remained constant.

Since February 1974 the patient has been treated with 2 mg melphalan per day.

Case Report 5

Female patient (EN) born 1895. The patient was previously in good health but was admitted to hospital for an acute gastroenteritis in October 1974. The ESR was 120 mm/hour and a serum M component of IgG type kappa was found. BJ protein was present in the urine. X-ray examination of the skeleton showed that multiple osteolytic foci were present in the skull, the clavicles and

the humeri. The plasma cells amounted to about 46 per cent of the nucleated cells in the bone marrow.

Melphalan and prednisone treatment was initiated but the patient died after 6 weeks. Autopsy findings were consistent with multiple myeloma.

RESULTS

Light Microscopy

In M-G-G stained smears the abnormal plasma cells showed a great variation in size and shape from patient to patient. The cytoplasm stained in different tones of blue to purple (Figs. 1 and 2).

The percentage of plasma cells in relation to the nucleated cells of the bone marrow, the percentage of plasma cells containing Rb in the total number of plasma cells, and the localization of these Rb are given in Table 1. The Rb were bluish or gray-blue and were often found together with unstained vacuoles. The number of Rb in a single plasma cell could vary from one to over a hundred (Fig. 1). When present in the nuclei the Rb always possessed a narrow dark brown rim (Fig. 3).

In patient OB a discrepancy is noted between the percentage of plasma cells with Rb obtained with the use of conventional M-G-G stained smears and that obtained by means of the immunofluorescence technique. This discrepancy can probably be explained by the presence of many very small Rb (0.1 μm) in the plasma cells of this patient. These small Rb cannot be visualized clearly in M-G-G stain, but are better resolved by fluorescence microscopy.

Whether situated in the cytoplasm or in the nuclei the Rb were always found to be PAS-negative (Fig. 4).

Fig. 1. M-G-G stain. A cluster of plasma cells is seen together with a metamyelocyte (arrow). One of the plasma cells contains over a hundred blue stained Russell bodies. Ca. 1000 ×

Fig. 2. M-G-G stain. A plasma cell in the metaphase stage of mitosis. Note the blue stained Russell bodies present among the chromosomes. A neutrophil myelocyte is seen in close contact with the plasma cell. Ca. 1260 ×

Fig. 3. M-G-G stain. Four plasma cells are seen together with a neutrophil leucocyte. One of the plasma cells has two intranuclear Russell bodies each with the characteristic brown rim. Ca. 800 ×

Fig. 4. PAS stain. A plasma cell with PAS negative intranuclear Russell bodies is seen together with a neutrophil leucocyte, the cytoplasm of which is positively stained with the PAS. Ca. 800 ×

Legend to Figs. 5 to 8

In all the immunofluorescence micrographs the staining for light chains is seen to the left and for heavy chains to the right.

Fig. 5. Extracellular Russell bodies (long arrow) are seen together with an intracytoplasmic Russell body (short arrow) in this preparation from patient EJ. Note the pronounced marginate fluorescence of the extracellular Russell bodies after treatment with TMRITC labelled anti-λ antiserum. In contrast, the intracytoplasmic Russell body cannot be depicted with this conjugate due to the strong fluorescence of the surrounding cytoplasm. This Russell body is more easily seen with phase contrast microscopy (right half part of the picture). Ca. 1000 ×

Fig. 6. A composite micrograph of the same plasma cell containing a Russell body in the perinuclear space (patient NN). Treatment with TMRITC labelled anti-λ antiserum (red) shows positive staining of the Russell body, while after treatment with FITC labelled anti-γ antiserum (green) the Russell body does not stain. The centr portion of the micrograph shows the Russell body (arrow) with phase contrast. Ca. 1000 ×

Fig. 7. A composite micrograph of the same type as Fig. 6 (from patient EN) shows plasma cell containing two intranuclear Russell bodies (arrow). Note positive fluorescence of the Russell bodies with TMRITC labelled anti-κ (red) and a negative result obtained after treatment with FITC labelled anti-γ antiserum (green). The centre portion shows both Russell bodies (arrow) with phase contrast. Ca. 1000 ×

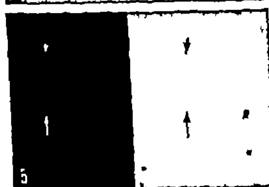
Fig. 8. A fluorescence micrograph showing a cluster of three intranuclear Russell bodies (arrow) in a cell from patient EH. A strong fluorescence is seen after treatment with TMRITC labelled anti-κ (red) and also after treatment with FITC labelled anti-κ (green). In both cases the fluorescence is particularly pronounced in the nuclear area adjacent to the Russell bodies. Ca. 1000 ×



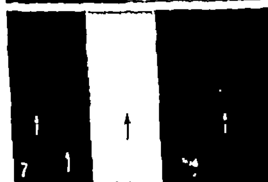



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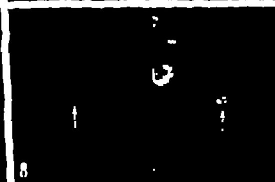

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TABLE 1 *Light Microscopy on Bone Marrow Aspirates and Immunochemical Characterisation of M Components in Serum from Patients with Multiple Myeloma*

Patient age/sex	Serum M component and conc. (g/litre)		Percentage of plasma cells in nucleated cells	Percentage of vacuolated plasma cells without Rb‡	Percentage of plasma cells with Rb in		Bence Jones (BJ) protein
					nucleus	cytoplasm	
EJ 45/F	IgG λ	81.6	65	7	5	12	+†
EH 61/F	IgA κ	≧43.9	24	17	3	1	—
NN 57/F	IgG λ	7.9	8	2	1	30	—
OB 45/M	BJ protein κ	≧ 1.0	8	53	1	4	+
EN 78/F	IgG κ	n.d.$	46	0	25	0	+

* At time of bone marrow biopsy
‡ Russell bodies.
† The sign + denotes the finding of Bence Jones protein in the urine
$ Not done.

Electron Microscopy

The plasma cells with Russell bodies showed an apparent discrepancy between the immature appearance of the nucleus and the mature appearance of the cytoplasm. The nuclei were large, rounded or ovoid with one or two nucleoli and the chromatin appeared cloudy and homogeneous (Fig 10) instead of the "cartwheel-like" appearance of chromatin seen in normal mature plasma cells. The shape of the plasma cells was often very irregular and the cells showed pseudopodial or microvilli like extensions of the cytoplasm (Fig. 10) The rough endoplasmic reticulum (ER) was very extensive and all types (19) ranging from the lamellar type to the globular type were seen (Figs. 9 10 14)

The Golgi complex was large and many vesicular elements were found in this region frequently together with a pair of centrioles. Mitochondria with well preserved cristae were present in the cytoplasm and so were "dense bodies" surrounded by a smooth triple layered membrane.

Cytoplasmic Rb were found only within the cisternae of the rough ER. Sections of about 300 plasma cells with Rb have been studied in detail, and the Rb were found to be of different shapes and sizes. Most often they were situated in the cytoplasm, but some were also present within the nucleus and individual plasma cells often had Rb in both regions (Fig 10) The majority of the cytoplasmic Rb were round, dense, homogeneous globules and no ordered or crystalloid substructure was ever found in their interior The surfaces of the bodies were usually smooth and round and always showed a clear separation from the surrounding medium which consisted of a moderately dense material similar in appearance to that seen in the other cisternae of the rough ER (Fig 9) The diameter of the bodies varied from 0.05 µm to 20 µm, and often several small Rb were observed within one and the same cisterna.

In one patient (EJ) some of the plasma cells contained aggregates of Rb of different size within the same dilated cisterna of the rough ER. These aggregates generally showed a characteristic pattern with a large central Rb surrounded by many smaller Rb which appeared as if they were in a process of coalescence with the large Rb. This type of aggregation we have called the "Medusa form (Figs. 12, 14 15)

Intranuclear Rb were found in the plasma cells of all the patients (see Table 1) Sections of 40 plasma cells with intranuclear Rb were studied in detail. In every case the intranuclear Rb were found in a vacuole surrounded by a triple layered membrane (Fig. 11) In addition to the Rb, the vacuoles also



Fig 9 A Russell body (Rb_1) is present in the dilated cisterna of the rough endoplasmic reticulum (RER) Part of another Russell body (Rb_2) is seen in the cisterna of an adjacent part of the RER. Limiting membranes cannot be seen around the Russell bodies. Ribosomes (R) are seen attached to the cytoplasmic side of the endoplasmic membrane. CM denotes cell membrane 90,000 ×

Figs. 9 to 15 are electron micrographs obtained from sections of human plasma cells containing Russell bodies. The bar on each micrograph represents 100 nm unless otherwise stated.

contained an amorphous, granular material of the same appearance as the contents of the cisternae of the rough ER. In a number of cells some membrane fragments and small membrane bound vacuoles were present in the intranuclear vacuole which also contained the Rb (Fig. 11) Both of the two morphological types of Rb described, i.e. the smooth and the "Medusa" forms, were found in the nuclei. The nucleoli were clearly separated from these inclusions and showed the same morphology as in plasma cells without Rb, whereas in some cells the chromatin appeared to be condensed in a rim around the inclusion membrane (Fig. 13)

In some plasma cells a Rb was found in the perinuclear part of the rough ER, which was dilated only in the region which contained this Rb (Fig. 12) Other plasma cells showed a Rb lying in a dilated portion of the perinuclear space which protruded into the nucleus at this location (Fig 13) A stage where the entire Rb was situated within the nucleus, but remained directly connected to the perinuclear space, has also been observed.

Immunofluorescence Studies

The percentage of plasma cells that stain specifically for H and L chains in the cytoplasm by the immunofluorescence technique is presented in Table 2. It is apparent that the percentage of M component containing cells of the total number of immunoglobulin

TABLE 2. *Immunofluorescence Studies on Bone Marrow Aspirates from Patients with Multiple Myeloma*

Patient	Percentage H and L chain containing cells of monoclonal cells					Percentage monoclonal cells of nucleated cells	Percentage Rb containing cells of monoclonal cells	Types of immunoglobulin chains detected in Rb
	H (Heavy)			L (Light)				
	α	μ	γ	κ	λ			
EJ	0	0	100	0	100	76	26	λ
EH	91	5	4	99	1	56	5	κ
NN	1	1	98	1	99	13	32	(γ)λ
OB	17	22	61‡	97	3	30	90†	κ
EN	1	0	99	99	1	5	14	κ

* Russell bodies.
‡ Very few of the monoclonal cells from this patient stained for H chains.
† Approximate number only difficult to distinguish between Rb and vacuoles.

containing cells is very high for all patients, *which is an indication of a malignant plasma* cell proliferation (11) Compared to the conventional M-G-G staining (see Table 1) the immunofluorescence technique is a more efficient method of depicting the monoclonal cells, especially for patients whose myeloma cells show a pronounced atypical morphology (patients OB and EH) Recognition of Rb by immunofluorescence is rather difficult, but combined with phase contrast microscopy it is possible to recognize them more precisely (Figs. 5 6, 7) However in instances where the Rb and the vacuoles containing them are very small (patient OB) and therefore difficult to resolve their identification can still be a problem. As seen in Fig 5 the fluorescence of the Rb is localized only on their periphery and may be difficult to distinguish from the *fluorescence of the surrounding cytoplasm* (patients EJ NN OB) or the fluorescence of that part of the nucleus which is in close contact with the Rb (patient EH) (Fig 8) Therefore in each preparation it was important to find some free extracellular Rb or some Rb which were situated in non-fluorescent surroundings (Fig 5) in order to secure a proper identification of their H and L *chain content. In this way only L chains were* detected in the majority of the Rb of the monoclonal cells of four of the patients, although H chains were expected to be present in the Rb of three of them (EJ EH EN)

In one patient (NN) both L and H chains *were found in most Rb of the cells, but also* for this patient many Rb stained positive for L chains only (Fig. 6) For patient EJ a few plasma cells were found to contain only λ chains in their cytoplasm as well as in the Rb. The cells of patient EH were peculiar because they showed a halo of strong fluorescence around the nuclear Rb. This halo showed a positive staining reaction for both *α and κ chains (Fig 8)*

DISCUSSION

The morphology of Rb as depicted by the light and electron microscopy studies of the present report is in accordance with results

Fig 10 Part of a plasma cell with an intranuclear and a cytoplasmic Russell body (Rb) Note the pseudopodial or microvilli-like extensions of the cytoplasm (MV) and the cloudy homogeneous appearance of the chromatin in the nucleus (N) A well developed Golgi complex (G) with a centriole (C) is also seen. 15,000 ×

Fig 11 Detail from Fig. 10. A part of the intranuclear Russell body is seen together with a membrane bound acuole (VA) also present in the nuclear vacuole, which otherwise is completely filled with the Russell body (compare Fig. 10) Note that the nuclear acuole is limited by a triple layered membrane (arrow) The centriole (C) and small vesicles (V) of the Golgi complex are also present. 90,000 ×

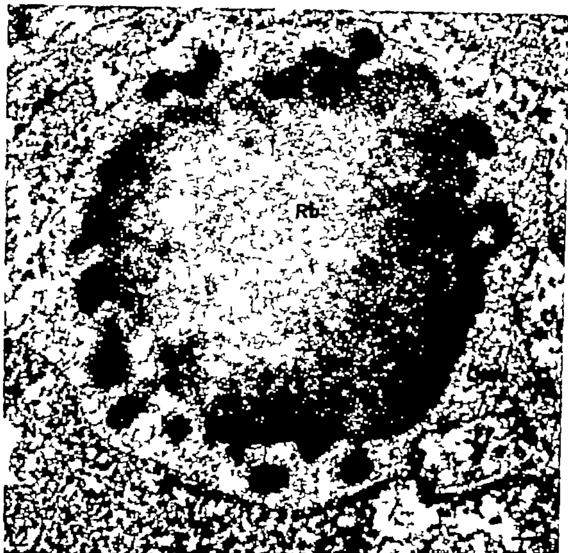


Fig. 12. A detail which shows a Russell body of the "Medusa" form situated in dilated region of the perinuclear space. Note the outer nuclear membrane (ONM) with ribosomes attached to its cytoplasmic side. 90,000 ×

previously published by *Maldonado et al.* (19) similar to these authors we found that the Rb in general were dense and homogeneous (Fig. 9).

Our data clearly show that a demonstration of Rb in plasma cells of the bone marrow of a patient has no obvious correlation with the heavy chain class or light chain type of the M components present in the serum and cells of the patient. However the patient (NN) constantly showed a low level of monoclonal IgG in serum samples and a small percentage of abnormal plasma cells in the bone marrow. These findings could indicate that the patient is in a premyeloma stage (29).

The myeloma cells with Rb seldom account for more than a few per cent of the total myeloma cell population (3). The most common form of a Russell body found in our study was a dense homogeneous globule (Fig. 9) the diameter of which could vary from 0.05 μm to 20 μm, the smallest being visible only in the electron microscope. Furthermore,

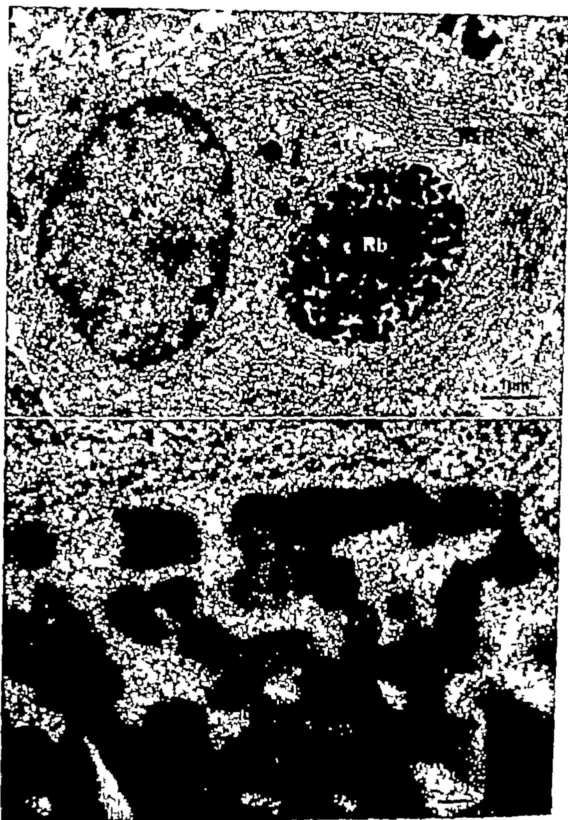


Fig. 13 Two Russell bodies are present in a dilated area of the perinuclear space. Note that the Russell bodies seem to have forced the inner nuclear membrane (INM) to protrude into the nucleus (*N*). Note also the rim of condensed chromatin around the protrusion. 90,000 ×

the smaller Rb sometimes had fuzzy outlines, whereas the larger ones were sharply demarcated.

Ultrastructural studies of consecutive bone marrow specimens taken during a period of 3 years were carried out for all patients except for patient EN from whom we only obtained marrow smears from one biopsy. During the observation period the morphology of the Rb and the number of cells with Rb remained unchanged in the individual patients and the Rb were found in specimens taken prior to, as well as after treatment of the patients with melphalan or cyclophosphamide.

In the plasma cells of one patient (EJ) Rb of the "Medusa" form were found (Figs. 12, 14, 15). Like other Rb they were found both in the cytoplasm and in the nuclei of the cells. This special configuration could probably represent an intermediate stage in the coalescence of smaller Rb into one or two bigger as suggested by *Brittin et al.* (5) and *Maldonado et al.* (19). This intermediate stage was presumably found more easily in


Rb
1μm

the cells of this particular patient because this patient also had a high percentage of plasma cells which contained Rb.

Two types of intranuclear inclusions in plasma cells have been described previously. The first is a membrane bound vacuole containing material of low density to electrons (8, 10, 21, 27). This type is considered irrelevant to the present study and will not be discussed further. The second type is also membrane bound, but contains electron dense material and presents a morphology similar to Rb (1, 5, 19, 26) (Figs. 10, 11). In our study this second type of inclusion has been found only in a small proportion of the abnormal plasma cells of the four patients studied by electron microscopy (EJ, EH, NN, OB).

Two different theories have been put forward concerning the origin and production of these Rb. *I*) Rb develop within the nucleus as a result of an intranuclear protein synthesis and subsequently become surrounded by a unit-membrane. Later the Rb are discharged into the perinuclear space and follow from here on the usual secretory pathway via the rough ER (1, 5, 8, 21, 27) or *II*) Rb are mainly produced in the cisternae of the rough ER, but some develop in the perinuclear space. A protrusion into the nucleoplasm of that part of the perinuclear space containing the Rb and a later pinching off of the inner part of the nuclear membrane could lead to the formation of an intranuclear Rb limited by a single unit membrane (4, 10, 19).

The results of our observations on intranuclear Rb in plasma cells tend to support the second theory. Forty different cells with intranuclear inclusions were studied. The vacuoles in which the inclusions were found were always limited by a single unit membrane and in several vacuoles some debris, presumably of cytoplasmic origin, was present together with the Rb (Fig. 11). Finally we have been fortunate to find sections in which what appears to be the transitional step in the passage of a Rb from perinuclear space to the interior of the nucleus can be seen (Figs. 12, 13). This intermediate stage is probably of very short duration as we have only encountered this particular location of a Rb a few times. Thus, all our evidence indicates that the intranuclear bodies are Rb which originally were formed in the perinuclear space of the cell.

Rb in cells from the five patients examined were all PAS negative, irrespective of whether the bodies were present in the nuclei or cytoplasm of the plasma cells and irrespective of the H and L chain type synthesized. This is in accordance with the findings of *Maldonado et al.* (19).

Immunofluorescence studies of plasma cells with Rb or inclusions with similar morphology are of limited number at present (5, 13, 20, 26, 30). *Ortega et al.* (20) and *Brittin et al.* (5) demonstrated, with the use of specific rabbit antisera to human γ globulin and human IgG respectively, a characteristic strong marginate fluorescence around the unstained cores of Rb in plasma cells obtained from human lymph nodes and bone marrow aspirates. Both groups concluded that Rb consist of accumulated immunoglobulins.

Hurez et al. (13) reported on a patient with chronic lymphatic leukaemia, who after 3 years developed vertebral osteolytic lesions and a simultaneous kappa Bence Jones proteinuria. In bone marrow smears a small percentage of plasma cells was found, all of which contained intracytoplasmic inclusion bodies, whereas no intranuclear inclusions were seen. The inclusion bodies stained pink with the Giemsa stain but remained unstained after PAS staining. Ultrastructural studies revealed medium electron dense bodies in the cisternae of the rough ER of the plasma cells, thus presenting characteristic features of Rb.

Fig. 14. Part of a plasma cell with a giant Russell body of the "Medusa" form. The rough endoplasmic reticulum (RER) of the cell is well developed and of the lamellar type (19). 15,000 ×

Fig. 15. Detail from Fig. 14 showing part of the periphery of the "Medusa" Russell body. Smaller Russell bodies appear as if they were in a process of coalescence with the more central part of the Russell body. 40,000 ×

Immunofluorescence studies showed that the Rb were stained by anti μ as well as by anti-kappa conjugates.

Stavem et al. (26) described intranuclear inclusions in plasma cells from a patient with multiple myeloma. These bodies showed ultra structural characteristics comparable to the intranuclear Rb we described in the present paper *Stavem et al.* also found that the inclusion bodies when stained with fluorescein-labelled anti-IgG showed a positively stained rim and an unstained core. They concluded that the electron dense inclusions did not bind anti-immunoglobulin either because 1) the inclusions did not contain immunoglobulin or 2) the immunoglobulin was denatured during preparation of their smears, or 3) the conjugate was unable to penetrate into the core of the bodies.

A patient with a non-secretory myeloma was described by *Hücher et al* (30) In the plasma cells of this patient cytoplasmic and intranuclear inclusions were found, which stained blue in the M-G-G stain. Electron microscopy showed that the electron dense cytoplasmic inclusions were situated within the cisternae of the rough ER. Based on these criteria we could classify these inclusions as Rb Fluorescent staining was achieved by an indirect staining method with monospecific rabbit antisera to human immunoglobulins and fluorescein conjugated swine anti rabbit IgG The cytoplasm of the plasma cells showed fluorescence with anti-γ and anti-κ, but the intranuclear inclusions did not show fluorescence. It was concluded that these inclusions presumably contained neither intact immunoglobulin nor free light chains. A fluorescence of the cytoplasmic inclusions was not described.

In all cases reported here we found a positive marginate staining of the Rb whether present in th cytoplasm or in the nuclei of the plasma c lls, when anti L chain conjugates were used Only one patient (NN) had Rb which also st ined positive with the antiserum to H chain However this patient may suffer from a different disease than myeloma, or be in a premyeloma stage since the M component concentration has remained constant for a long period and osteolytic foci have not appeared.

With normal regulation of immunoglobulin production in plasma cells a balanced H and L chain synthesis usually occurs which eventually leads to secretion of normal tetra chained molecules (2 25) However plasmocytoma cell lines may produce excess amounts of L chains probably due to defective control mechanisms (2) The L chain surplus may be secreted in the form of free monomer or dimer L chains, *i.e* the Bence Jones proteins.

In our studies of plasma cells from patients with multiple myeloma the findings could indicate that a hyperproduction of L chains in combination with a secretory abnormality of the plasma cells could lead to an accumulation of L chains and thus to the formation of Russell bodies in the cells.

The excellent technical assistance of Mrs. *Jytte Berg* Mrs. *Anne Grethe Caspersen* and Mrs. *Ulla Holby* is gratefully acknowledged. Mr *Finn Larsen* and Mrs. *Anne Grethe Overgaard* are thanked for their expert photographic work.

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BRIEF REPORTS

HETEROTRANSPLANTATION OF A HUMAN MAMMARY CARCINOMA TO THE MOUSE MUTANT NUDE

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Spang-Thomsen, M. Heterotransplantation of a human mammary carcinoma to the mouse mutant nude. Acta path. microbiol. scand. Sect. A, 84 350-352 1976

A human mammary carcinoma was heterotransplanted to athymic nude mice. Viable tumour tissue was maintained throughout the observation periods which, because of the limited lif span of nude mice, were relatively short In one animal surviving for a longer period, actual tumour growth was observed. It is concluded that human mammary carcinoma is capable of growing in nude mice but, because of the frequently low growth rate of these tumours, prolonged periods of observation are required in order to reveal such growth.

Key words: Heterotransplantation mammary carcinoma, human nude mice.

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The mouse mutant nude suffers from congenital thymic aplasia (5) The resultant immunological incompetence permits acceptance of wide range of heterografts. In particular the number of transplanted human malignant tumours is increasing steadily (3 7 8, 9 11 12, 14)

The model system, nude mice with transplanted human malignant tumours, appears to be the most promising in studies of antineoplastic agents (4 15) and in such studies mammary carcinomas are *extremely relevant objects. Transplantation experiments* with this type of tumour have hitherto been unsuccessful t being possible only to maintain viable tissue, but not to detect actual tumour growth (6, 13)

In this report a successful experiment is described during which a human mammary carcinoma presented unmistakable growth after being transplanted to nude mice. In this connection, the reasons for failure in previous experiments are discussed.

Material and Methods

Mice The experiments were made on 10 female SPF nude mice, 6 weeks old, of BALB/c origin, obtained from the Laboratory Animals Breeding and Research Centre, Gl. Bomholtgård, DK-8680 Ry Denmark. The animals were kept in laminar sterile air flow clean benches. Room temperature 25 ± 2 C. Relative humidity: 55 ± 5 per cent. Sterile water and autoclaved food pellets were given *ad lib*

Tumour The tumour tissue was obtained from a 56-year-old woman in the 7th year after her menopause She had never undergone hormone treatment. There was no history of gynaecological diseases, but on two occasions since *1971 fibrocystic* disease of the breast had been observed.

The patient found the tumour herself 6 weeks before the operation. It was situated in the upper lateral quadrant of the left breast, 3 cm from the nipple. It measured 3 × 2 cm and was not connected to skin or underlying tissue During surgery (Record No. Z 11.685 Finsen Institute surgical dept.) and apparently viable biopsy specimen of about 1 cm was removed and immediately brought to the laboratory in phosphate-buffered saline containing penicillin and streptomycin. Hypoxia did not persist for more than 14 to 40 minutes

Histological examination of sections adjacent to the graft and of the tumour removed during sur-

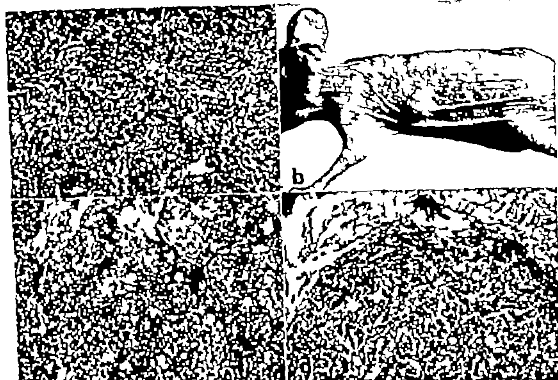


Fig. 1 a. Primary human tumour material, solid mammary carcinoma. Haematoxylin-eosin. × 108

Fig. 1 b. Tumour growth in mouse No. 8, 175 days after grafting.

Fig. 1 c. Histology of tumour shown in Fig 1 b. Haematoxylin-eosin. × 108.

Fig. 1 d. Maintained viable tumour tissue in the dorsal subcutis of mouse No. 9. Haematoxylin-eosin. × 108.

gery showed tumour tissue of identical structure solid carcinoma, grade II (WHO CII)

Transplantation. This was performed under general anaesthesia with 0.25 ml propanidid (®Epontol). Blocks of tissue, 3 × 2 × 2 mm in size were transplanted either subcutaneously in the flanks, as described by *Povlsen & Rygaard* (11) or in the fourth mammary fat pad.

The latter implantations were performed by making a V-shaped incision in the distal abdominal wall, and the skin flap was then reflected during blunt dissection. The tumour inocula were inserted in pocket preformed in the fat pad using blunt instrument.

Observation and autopsy. The animals were examined three times a week with a view to revealing tumour growth. In all animals autopsy was performed, except in two where decomposition was so pronounced that proper examination was not feasible. During autopsy all viscera were examined, and those presenting gross changes were removed for histological examination, together with tumours or skin with underlying tissue corresponding to the sites of grafting.

Histology. The tissue specimens were fixed in formalin, embedded in paraffin, and sections of 4 μ were stained with haematoxylin-eosin or by the van Gieson-Hansen method.

Results

Nine of the ten animals died between the 28th and 130th day after transplantation, and in these cases no definite tumour growth could be detected.

In mouse No. 8, unmistakable growth was observed on the 138th day (Fig. 1 b) on the site of inoculation in the left flank. This tumour grew slowly from 25 mm³ to 65 mm³ on the 178th day when the animal had to be killed because of wasting. Before that occurred, 8 inocula were serially transplanted to 4 mice and adjacent tissue was removed for histological examination. During the period when the tumour growth could be followed, the doubling time was approx. 30 days.

Histologically the tumour maintained its original structure and cytological characteristics (Figs. 1 a and c)

Examination of the sites of inoculation in the other animals revealed viable tissue with identical characteristics in three fat pads inocula and four subcutaneous inocula (Fig. 1 d)

Benign mammary tissue (ducts) was accidentally transplanted in some grafts, and this tissue was also accepted. Furthermore areas with fat necroses were found in some cases, presumably originating from transplanted human fatty tissue.

Metastases were never observed.

At the time of death, all the animals showed signs of wasting, presumably resulting from the well-known liver disease in the nude mouse (2, 10)

Discussion and Conclusions

In allotransplantation experiments with *murine mammary tissue* the fourth mammary fat pad was found to be a better site of grafting than the subcutis of the flanks (1) In previous heterotransplantation experiments, where attempts were made to transplant *human mammary carcinoma* to nude mice, the grafting was made in the dorsal subcutis. Since no tumour growth, but only maintenance of viable tissue, was detected in these studies (6, 14) it was decided in the present experiment, to use both the mammary fat pads and the subcutis of the flanks as inoculation sites.

The results of the present study do not support the assumption that the mammary fat pads in the nude mouse are more suitable than the dorsal subcutis as sites for transplanting human breast carcinoma.

As regards the animals dying at a relatively early stage, the present study confirms the results of previous heterotransplantation experiments, maintained viable tissue being found in these animals. However in the animal which survived for a considerably longer period, tumour growth was observed, with a doubling time of 30 days. Assuming that growth was approximately exponential, extrapolation to the time of grafting shows the volume of the surviving part of the graft to be about 1 mm³ This is in full accordance with the conditions of the types of tumours growing more rapidly in nude mice.

It must be concluded that human mammary cancer is capable of growing in nude mice and the only reason why previous investigators have not detected any growth must be that the periods of observation have been relatively too short.

Although human mammary carcinoma is thus capable of growing in nude mice, studies on antineoplastic agents will most likely present difficulties because of the frequently very low rate of growth of these tumours.

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UROTHELIAL HYPERPLASIA OF THE RENAL PAPILLAE IN FEMALE SPRAGUE-DAWLEY RATS INDUCED BY LONG TERM FEEDING OF PHENACETIN

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Johansson, S. & Angervall, L. Urothelial hyperplasia of the renal papillae in female Sprague-Dawley rats induced by long term feeding of phenacetin. Acta path. microbiol. scand. Sect. A, 84 353–354 1976.

Twenty-one of 40 rats fed phenacetin in the diet for up to 86 weeks developed urothelial hyperplasia of the renal papillae. Two of 30 rats in the control group had similar changes associated with chronic pyelitis. The difference is statistically significant ($p<0.01$)

Key words: Urothelial hyperplasia phenacetin.

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An association between high intake of phenacetin-containing drugs and development of renal pelvic tumours has been found (*Hultengren et al.* 1965 *Bengtsson et al.* 1968, *Angervall et al.* 1969 *Johansson et al.* 1974) This association has been further supported by findings in studies of the metabolism of phenacetin (*Nery* 1971) The aim of the present investigation was to study the effects of orally administered phenacetin on the epithelial lining of the renal pelvis of rats, in order to obtain further evidence of the suggested carcinogenic effects of this drug.

Materials and Methods

Fourty female Sprague-Dawley rats fed 0.535 per cent phenacetin in the diet were used for the experiment. A control group consisting of 30 female Sprague-Dawley rats were fed ordinary rat pellets (Astra-Ewos) without phenacetin. The rats had free access to food and tap water The rats were weighed and food consumption was determined every week during the experiment. The rats were sacrificed after 86 weeks. Post mortem examination of all rats was performed. Organs with grossly visible pathological changes were examined histologically The kidneys from all rats were weighed and fixed in 10 per cent neutral formalin solution. The urinary bladder was inflated with and fixed in 10 per cent neutral formalin solution. Five µm thick sections were stained with haematoxylin-eosin and haematoxylin-van Gieson. The urothelial hyperplasia and the vascular changes of the renal papillae were graded as no changes, mild, moderate, and severe. Statistical calculations were performed as trend in a contingency table which is a special variation of Fischer's non-parametric permutation test (*Odén & Wedel* 1975)

Results

The food consumption of the control rats and rats fed phenacetin did not differ The average body weight of the control rats was highest, varying between 10–35 grams during the experiment, except at the end of the latter when it was identical in both groups, namely 290 grams. There was no statistical significant difference in the mean kidney weight of animals in the two groups.

Six control rats and 2 rats fed phenacetin died early during the experiment and due to severe autolysis, histological examination was not performed. Hyperplasia was of severe degree in 6 of the other rats fed phenacetin while mild to moderate hyperplasia of the urothelium covering the renal papillae was manifest in 15 In 3 rats, hyperplasia was found to involve almost the whole papillae and it was focal in 18 rats. In 6 rats, the urothelial hyperplasia was papillary being flat in

15 (Fig. 1) The hyperplasia found in the rats fed phenacetin was not associated with pyelitis except in 1 case. In 1 control rat, severe hyperplasia of the urothelium was associated with severe pyelitis, and in another control rat mild to moderate unilateral epithelial hyperplasia of the renal papillae associated with moderate pyelitis was found. In 5 of the rats fed phenacetin severe dilatation of the vasa recta was observed and in 10 mild to moderate dilatation was associated with the urothelial hyperplasia. The difference in frequency and degree of the hyperplastic and vascular changes in the control rats and the rats fed phenacetin was statistically significant ($p<0.01$). The urothelial hyperplasia was associated with dilated vasa recta, often also with focal interstitial calcification, but no apparent papillary necrosis or interstitial nephritis was demonstrated. In 2 control rats, a few dilated vasa recta were found.

Discussion and Conclusion

Urothelial hyperplasia of the renal papillae has not been found in normal or ageing rats (*Lalich et al.* 1974). However urothelial hyperplasia of the renal papillae has been induced by excess of sodium chloride or addition of monosodiumglutamate to the diet in uninephrectomized rats (*Lalich et al.* 1974). The authors provided no explanation of the epithelial hyperplasia since, in contrast to the present experiment, multiple factors were employed simultaneously on the rats.

The results of the present investigation lend strong support to the opinion that phenacetin has carcinogenic or cocarcinogenic properties. Our findings have initiated a long-term study (over 2 years feeding of phenacetin) and such an experiment is now about to be terminated.

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Fig 1 Urothelial papillary hyperplasia of the renal papillae associated with a dilated vasa recta in a female Sprague-Dawley rat fed phenacetin for 86 weeks. Note the focal calcification at the base of the papillae.

REPAIR IN ARTERIAL TISSUE. ELECTRON MICROSCOPY OF EVANS BLUE VITAL STAINED EMBOLECTOMY CATHETER LESION OF THE RABBIT THORACIC AORTA

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Collatz Christensen, B., Chemnitz, J. & Tkocz, I. Repair in arterial tissue. Electron microscopy of Evans blue vital stained embolectomy catheter lesion of the rabbit thoracic aorta. Acta path. microbiol. scand. Sect. A, 84 355–357 1976.

The rabbit thoracic aorta was studied by EM and vital staining with Evans blue at varied points of time after a single lesion produced by an embolectomy catheter. EM of white areas: the surface cells of the myo-intimal thickening resembled endothelial cells with a discrete occurrence of microfilaments inside the plasma-membranes and rather differentiated flap-like junctions. EM of blue areas: the surface cells resembled modified smooth muscle cells with a heavily contrasted zone just inside the luminal plasma membrane containing closely packed microfilaments. The cell contacts either were missing, or they presented undifferentiated side-to-side contacts.

Key words: Electron microscopy; embolectomy catheter lesion; endothelium, Evans blue vital staining.

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In a current study vital staining with Evans blue of the thoracic aorta following a single dilatation trauma has been correlated with cellular patterns in the intimal surface as revealed by perfusion silver-staining. The cellular patterns previously described and classified were reproduced (*Collatz Christensen & Garbarsch* 1973). The findings obtained by vital staining and silver staining correlated as follows. White areas: normal and hexagonal endothelium in continuity with endothelium of intercostal arteries and probably derived from the latter. Blue areas: immediately after the lesion denuded surfaces and, some days later pleomorphic patterns described as "foam-like patterns" and considered pseudoendothelium i.e. derived from sources other than endothelial cells surviving the trauma. In the present report, the question is challenged whether ultrastructural differences between white and blue areas also may exist.

Materials and Methods

Seven male albino rabbits of the Danish country strain, 2.5–3.0 kg, were used. 3 were sacrificed 8 days after the lesion, 1 after 14 days, 1 after 21 days, and 2 rabbits served as controls. The lesion was produced by an embolectomy catheter introduced to the level of the aortic arch via the left femoral artery and retracted with inflated balloon till resistance at the diaphragm. *Vital staining*: Evans blue 0.5 per cent intravenous in an ear-vein 4 h before autopsy. Perfusion fixation using buffered glutaraldehyde was performed according to methods previously described (*Collatz Christensen & Garbarsch* 1973, *Collatz Christensen* 1974). Standard procedure for EM preparation: selective excision of specimens from white and blue

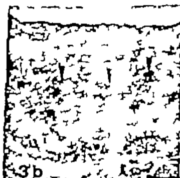




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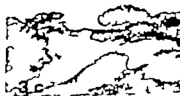


Fig. 1 Aorta vital stained with Evans blue 8 days after a lesion produced by embolectomy catheter. Note the white areas around intercostal arteries.

Fig. 2 a–b EM from white intimal area. a) Contacts between surface cells are rather extensive. Nucleus projects into the lumen. Endocytotic vesicles (arrow heads) (× 10,800) b) A higher magnification of the cell-to-cell contact resembling endothelial flaps seen in Fig. 2 a. Note the desmosome-like structures (arrows) (× 24,800)

Fig. 3 a–c EM from blue intimal area. a) The cytoplasm of a surface cell containing a prominent dilated RER. Note the heavily contrasted zone inside the luminal plasma membrane (arrows) (× 6,800) b) A higher magnification of the heavily contrasted luminal zone. This zone probably contains closely packed microfilaments with spotty appearance of condensed streaks (arrow heads) (× 26,000) c) In this micrograph no obvious, direct contact between the surface cells is visible. (× 14,000)

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areas, immersionfixation in buffered glutaraldehyde, postfixation in osmium, contrasting en bloc with uranyl acetate dehydration in acetone, embedding in Araldite. Ultrathin sections were contrasted with the uranyl acetate and with lead citrate.

Results and Comments

The gross appearance of vital stained aorta 8 days after the lesion is presented in Fig. 1 EM from white intimal areas: Intima was moderately thickened and covered by flat cells with nuclei prominent towards the lumen. The cytoplasm of the surface cells contained several endocytotic vesicles and caveoli in a luminal position, a dilated rough endoplasmic reticulum (RER) a well developed Golgi complex and dispersed mitochondria (Fig. 2 a). In several cells from animals sacrificed 8 days after lesion, a narrow partly interrupted zone just inside the abluminal plasma membrane as filled with a discretely contrasted substance apparently formed by microfilaments. In experiments based on 14 and 21 days' observation the surface cells contained more fibrillar substance now also oriented in a narrow continuous layer just inside the luminal plasma membrane. Cell contacts were rather extensive side-to-side contacts of plasma membranes with sites presenting denser contrast and desmosome-like structures. At the luminal surface, overlapping cell borders frequently formed areas resembling endothelial flaps (Fig. 2 b)

EM from blue intimal areas: The intima was thickened and covered with voluminous cells (Fig. 3 a) The cytoplasm of surface cells contained a prominent dilated RER and Golgi complex, whereas endocytotic vesicles were rare (Fig. 3 a, b) In several surface cells the most conspicuous feature was a broad zone free of organelles just inside the luminal plasma membrane (Fig. 3 a, b) This zone was heavily contrasted and seemed to consist of closely packed microfilaments with spotty appearance of condensed streaks (Fig. 3 b) The cell contacts were membrane contacts side-to-side of small extension, and often no direct contact was obvious (Fig. 3 c) Desmosome-like structures and areas resembling endothelial flaps were absent.

Subendothelial tissue: In white as well as in blue areas, the dominant cells resembled modified smooth muscle cells.

EM seemed to confirm differences in the morphology of intimal surface cells from white and blue areas. In white areas, some surface cells with structures resembling endothelial flaps and fibrillar substance inside the abluminal plasma membrane resembled "A-cells" described in 1975 by *Schwartz et al.*, who considered these cells to be modified endothelial cells. From blue areas, the surface cells described above were considered identical with the "C-cells" explored by *Schwartz et al.* 1975 and related to smooth muscle cells. It is open to discussion whether ultrastructural variations in the morphology of surface cells from white and blue areas of the intima can be causally related to the different permeability by Evans blue.

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PROLONGED SURVIVAL OF AKR MICE TREATED WITH THE SAPONIN ADJUVANT QUIL A

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Ebbesen P., Dalsgaard, K. & Heler Madsen, M. Prolonged survival of AKR mice treated with the saponin adjuvant Quil A. Acta path. microbiol scand. Sect. A, 84 258-260, 1976.

Treatment of AKR mice with the saponin Quil A delayed their death in spontaneous leukaemia. *In vitro* tests did not demonstrate any influence on infection of rat cells with Kirsten sarcoma virus. Quil A in the concentration nanogram/ml doubled the mitogen response of AKR spleen cells to lipopolysaccharide.

Key words Saponin Quil A survival of mice.

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Treatment of *leukaemic animals* and human subjects with the immun adjuvant BCG may enhance the survival time (*Mathé et al.* 1969 *Harun-Ghera & Weiss* 1973 *C ... et al.* 1973) The granulomas resulting from the use of BCG however limit its value. W therefore tested a saponin immune adjuvant, Quil A, for influence on the life span of AKR mice in which leukemia develops spontaneously (*Dunn 1954*) *Quil A has only* minor granuloma inducing property (*Dalsgaard* 1974)

The saponin Quil A was isolated from the cortex of the South-American tree *Quillaja ponaria Molina* (*Dalsgaard* 1974) From two months of age our inbred female AKR *mice* (*Ebbesen* 1974) *were monthly given one subcutaneous inoculation* of 50 µg Quil A in 0.5 ml water and were inspected six days a week. When ill, the mice were killed, and lung, liver spleen, kidney lymph nodes, cor subcutaneous tissue, thymus and thyroid were taken for microscopy the peripheral leucocyte count and haematocrit values were determined. Kirsten murine sarcoma *virus* (*Kirsten & Mayer* 1967) was used for *in vitro* infection of cells from normal rat kid ney Prior t infection, the cells or virus were pre treated by incubation with Quil A 2.5 µg/ml for *30 minutes at 37° C. Two-fold dilutions of virus* were used and the end point titre scored on day seven. The *mitogen* E. coli O55 B5 lipopolysaccharide (LPS) was obtained from Difco (lot No. 505309) and purified phytohaemagglutinin (PHA) from Wellcome (HA17 lot. No 8805) Eagle's minimum essential medium containing 10 per cent foetal calf serum (Flow lot No. CO553018) was *used for cell suspension made from spleens of eight* week-old untreated AKR mice 0.1 ml of cells + 1.8 ml of medium + 0.1 ml of mitogen and/or Quil A gav final concentration of 10^6 viable cells per ml. After 48 hours t 37 C, 4 per cent CO_2 ^{3}H thymidin 1 µCi/ml was added and the radioactivity of the cells determined 24 hours later

Any histological evidence of reaction to Quil A was not seen. Leukaemia developed and was considered the cause of death in all but one treated and one control mouse the mean *survival time however was enhanced by Quil A* (Table 1) Preincubation of target cells or Kirsten sarcoma virus with Quil A did not influence *in vitro* infection using this tumour virus Quil A alone had slight *mitogenic* activity and concentrations t or above 1 µg/ml were toxic to lymphocytes (T ble 2) With

TABLE 1 *The Influence of Substance Isolated from* Quillaja saponaria Molina *(Quil A) (50 µg sc/month) on AKR Mice*

	Number of mice	Leukaemic	Median survival time (months)	Range	Rank sum test*
Quil A treated	37	36	8.7	4–18	<0 01
Control (H_2O treated)	37	36	6.3	2–15	

	Mean leucocyte count ± SD	Mean weight in grammes ± SD	Mean haemoglobin ± SD	P
Quil A treated	22304 ± 27854	24 ± 3 8	55% ± 14	>0 1
Control (H_2O treated)	24500 ± 20994	24 ± 5	44% ± 13.5	

Mann-Whitney

TABLE 2. *The Influence of Quil A* In Vitro *Mitogen Response of AKR Spleen Cells Incubated with PHA or LPS*

Test numbers	Cells/ml	Mitogen/ml	Quil A/ml	x cpm ± SD
1–3	10^6	–	–	6 821 ± 1,975
4–6		1 µg PHA	–	35,944 ± 1 603
7 9		10 µg LPS	–	42,971 ± 2 422
10–12			1 µg	1,296 ± 526
13–15			0 1 µg	11 428 ± 1 138
16–18			0.01 µg	9,327 ± 1,036
19–21			0.001 µg	10 074 ± 1 419
22–24		1 µg PHA	1 µg	23,935 ± 2,027
25–27			0.1 µg	45,028 ± 1 145
28–30			0 01 µg	39,279 ± 3 629
31 33			0 001 µg	34,000 ± 2 478
34 36		10 µg LPS	1 µg	1,066 ± 580
37–39			0.1 µg	64 799 ± 3 845
40–42			0.01 µg	70 499 ± 2,983
43–45			0.001 µg	81,542 ± 7 641
46–48				256 ± 49

optimum concentrations of LPS and PHS respectively (*Haver-Madsen & Rubin* 1975) Quil A, at 1 nanogram/ml, doubled the response to LPS. Quil A influenced only slightly the mitogenic effect of PHA (Table 2)

To our knowledge other report on the influence of a saponin on the development of *leukaemia* has not been published. The *in vitro* test using Kirsten sarcoma virus did not indicate any influence of Quil A on non-immune tumour virus-cell interaction. It appears, however that Quil A has an enhancing effect on the activity of LPS, a mitogen known preferentially to stimulate B lymphocytes (*Möller* 1972) To our knowledge, no other compound influences the mitogenic effects of LPS in concentrations (nanogram/ml) as low as those sufficient for Quil A. The demonstrated adjuvant properties of Quil A could be responsible for the life prolonging effect on AKR mice

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pelse Daell Fonden Landsforeningen til Kræftens Bekæmpelse Statens lægevidenskabelige Forskningsråd and F L *Smidth & Co s Jubilæumsfond*

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ERRATUM

ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

Sect. A, 84 4 1976

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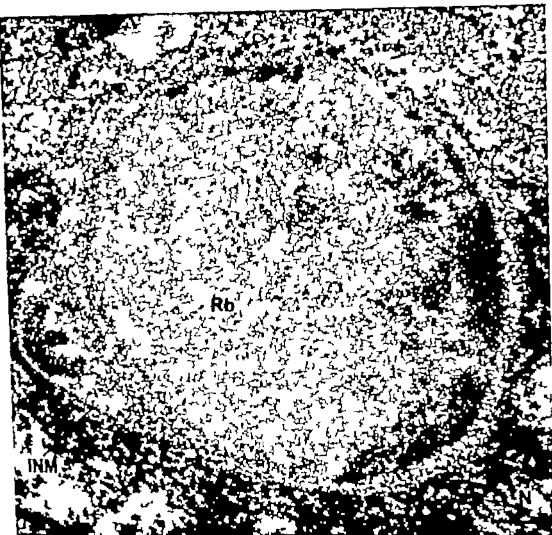
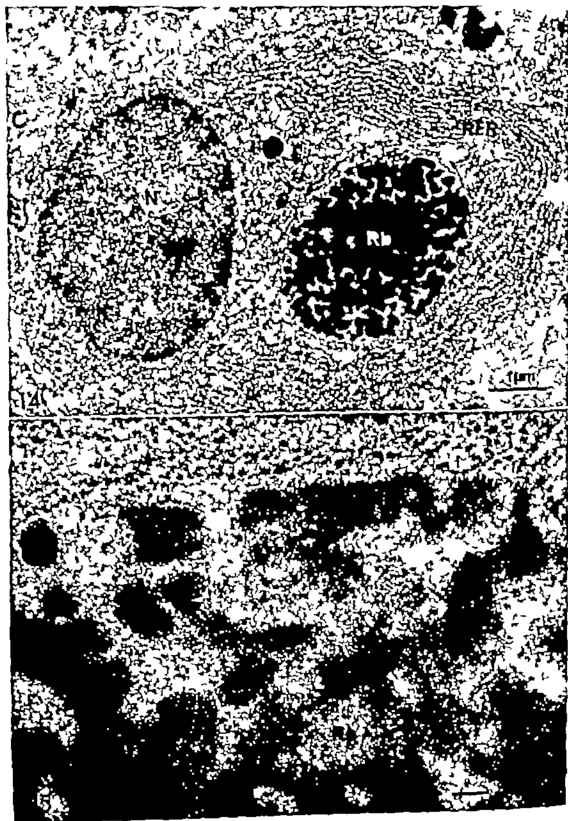


Fig. 13. Two Russell bodies are present in dilated area of the perinuclear space. N t that the Russell bodies seem to have forced the inner nuclear membrane (INM) to protrude into the nucleus (N) Note also the rim of condensed chromatin around the protrusion. 90,000 ×

the smaller Rb sometimes had fuzzy outlines, whereas the larger ones were sharply demarcated.

Ultrastructural studies of consecutive bone marrow specimens taken during a period of 3 years were carried out for all patients except for patient EN from whom we only obtained marrow smears from one biopsy During the observation period the morphology of the Rb and the number of cells with Rb remained unchanged in the individual patients and the Rb were found in specimens taken prior to as well as after treatment of the patients with melphalan or cyclophosphamid

In the plasma cells of one patient (EJ) Rb of the "Medusa" form were found (Figs. 12, 14 15) Like other Rb they were found both in the cytoplasm and in the nuclei of the cells. This special configuration could probably represent an intermediate stage in the coalescence of smaller Rb into one or two bigger as suggested by *Brittin et al.* (5) and *Maldonado et al.* (19) This intermediate stage was presumably found more easily in


C
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STUDIES OF TRANSITIONAL CELL TUMOURS OF THE BLADDER

Prognosis and Causes of Death

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Sjolin, K. E., Nyholm, K. & Trautner K. Studies of transitional cell tumours of the bladder. Prognosis and causes of death. Acta path. microbiol scand. Sect. A, 84 361–374 1976.

Transitional cell tumours of the bladder from a total of 228 patients were histologically classified as papillomas, papillomatous carcinomas and non-papillomatous carcinomas. Each group was subdivided into four grades of dysplasia. Papillomas and papillomatous carcinomas occurred in younger patients at a higher rate than non-papillomatous carcinomas. The 5-year-survival of patients with papillomas and carcinomas was 70 per cent and 26 per cent, respectively Among patients with papillomas with dysplasia grades 1 and the survival rate was almost identical. I the group of patients with papilloma thrombo-embolic diseases were the most common cause of death. Carcinoma of the bladder developed in about 30 per cent of the patients in this group. Recurrence of the papilloma only rarely changed the grade of dysplasia. If the recurrence was in the form of a carcinoma, an increase in the grade of dysplasia was common. The survival was more favourable among patients with carcinomas dysplasia grade 2 than among those with dysplasia grade 3 The rate of survival was higher in the group of patients with papillomatous carcinoma than among patients with non-papillomatous carcinomas. Among the deceased patients with primary carcinomas, 77 per cent died with carcinoma of the bladder To certain degree the grade of tumour cell dysplasia seems to be an expression of the malignancy of the tumours. The duration of the disease and the ppearance of tumours (papillomatous, non-papillomatous) may have relation to the patients defence system. At autopsy the papillomatous and non-papillomatous carcinomas were found to be similarly disseminated regardless of the difference in survival rate.

Key words Transitional cell tumours; bladders prognosis causes of death.

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The purpose of the present work is to examine the prognoses and causes of death among patients with transitional cell tumours of the bladder classified and graded in accordance with WHO's reference center (*Mostofi* 1968, *WHO* 1973)

MATERIAL AND METHODS

Our study is based on examination by way of light microscopy of formalin fixed tissue (biopsies and surgical specimens) stained with haematoxylin-eosin and van Gieson-Hansen.

W have previously applied these grades of dysplasia (*Sjolin & Hansen* 1972 and *Schroeder et al.* 1973): Dysplasia grade 1 where the cells are differ-

TABLE 1 228 *Tumours of the Bladder*

Papillomas 104 (95)	dysplasia, grade 0 2 dysplasia, grade 1: 70 dysplasia, grade 2 30 dysplasia, grade 3 2
Transitional cell	
Papillomatous 62 (61)	dysplasia, grade 1: 9 dysplasia, grade 2 37 dysplasia, grade 3: 16
Carcinomas 112	
Non-papillomatous 50 (48)	dysplasia, grade 1: 2 dysplasia, grade 2 18 dysplasia, grade 3: 30
Squamous cell carcinomas 11	
Adenocarcinomas 1	

Three of the figures are placed in brackets. These figures indicate the number of patients who were followed from the time when the first biopsy was obtained and are applied in Figs 1 4 and 5

In the other patients, 9 with papillomas, one with papillomatous carcinoma and 2 with non-papillomatous carcinomas, the histological diagnosis was primarily established on the basis of a recurrence.

changed ("the good ones) dysplasia grade 3 where the cells are most changed, anaplastic or ery polymorphic ("the bad ones") and grade 2 ("the in betweens") where changes are of medium degree. Papillomas with normal urothelium have been termed papilloma grade 0. In our work, only tumours with destructive invasion of adjacent tissue were termed carcinomas, whether they were papillomatous or non-papillomatous. The carcinomas were divided into Transitional cell carcinoma squamous cell carcinoma, and adenocarcinoma. *The transitional cell carcinomas were divided into* papillomatous and non-papillomatous, based on histological examination exclusively The transitional cell carcinomas were also graded according to the above mentioned three grades of dysplasia.

This classification and grading is pretty close t that suggested by *Broders* (1922) *Steinius* (1925) and The Institute of Urology (*Pugh* 1957) If compared with that established by *Bergkvist et al.* (1965) the only difference is that our dysplasia grade 3 corresponds t Bergkvist s grades 3 and 4

In some transitional cell tumours there may be minor areas of squamous or glandular metaplasia. In spite of this, the tumours would still belong in the group of transitional cell tumours.

Patients

The series comprises 234 patients with histologically verified tumours of the bladder in the period 1956–1970, inclusive. Six of these were excluded because of concomitant carcinomas or papillomas in the renal pelvis.

The histological diagnoses in the remaining 228 patients are presented in Table 1 The classification and grading is based on a revision of all primary biopsies and surgical specimens. In 19 patients, 10 with papillomas and 9 with carcinomas, the first histological diagnosis was made in another hospital. These diagnoses ha e been revised in our Institute of pathology

RESULTS

The age distribution applying to patients with transitional cell tumours, papillomas and carcinomas, respectively appears from Table 2 There was no difference between the age of patients and types of tumour

TABLE 2. *Ag Distribution among 104 Patients with P pillomas and 112 Patients with Transitional Cell Carcinomas in the Bladder*

	Papilloma 104	Carcinoma 112
<30	0	0
30–39	2	1
40–49	7	4
50–59	35	22
60–69	28	46
70–79	24	26
80–89	8	11
≥90	0	0

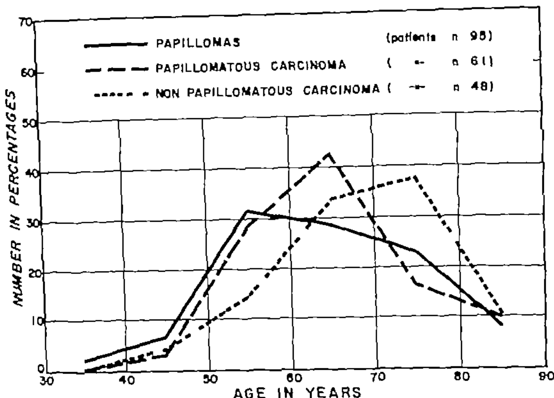


Fig. 1 Age distribution for patients with transitional cell tumours of the urinary bladder

(papilloma or carcinoma) ($p > 0.05$*) Nor was there any difference in the correlation between age and grade of dysplasia ($p > 0.1$*) Papillomas and papillomatous carcinomas were more frequent among younger patients, while the non-papillomatous carcinomas appeared predominantly in elderly patients ($p < 0.05$*) (see Fig 1) The 5-year cumulative survival among patients with papillomas and carcinomas treated with conservative surgery on the bladder was 70 per cent and 26 per cent, respectively (Fig 2) The difference is significant ($p < 0.0005$**)

Papillomas

This group comprises 104 patients (Table 1) The age distribution is demonstrated in Table 2. The sex distribution was male/female 85/19 The relation between age and grade of dysplasia 1 and 2 at the time of the first diagnosis appears from Fig. 3. No difference was demonstrated.

Fig 4 demonstrates the cumulative survival rates among patients with papillomas with dysplasia grades 1 and 2 respectively There is no significant difference between the groups ($p > 0.05$**) Two patients had a solitary papilloma, dysplasia grade 0 One a 70-year old man was controlled throughout two years during which period signs of recurrence were not observed, but he was not seen later The other a 58-year-old man is still under control and without recurrence after 7 years. As regards the two patients with multiple papillomas, dysplasia grade 3 one survived for less than one year He died from a pulmonary embolus to occur in connection with transvesical prostatectomy and electrocoagulation of the papillomas. At autopsy it was observed that the papillomas had invaded deep into the bladder wall. The other patient had also multiple papillomas and received supervoltage radiotherapy He died within two years after the primary diagnosis from multiple meta

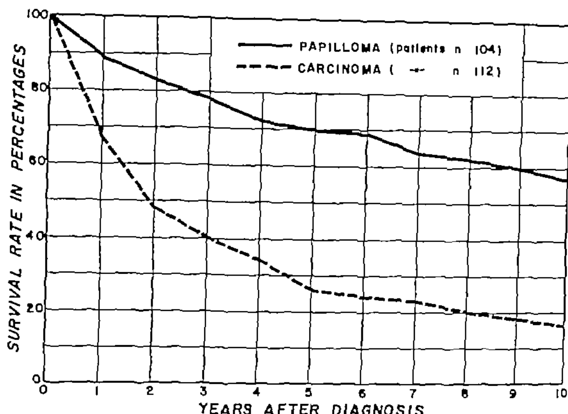


Fig 2 Cumulative survival curves for patients with transitional cell bladder tumours.

stases to pelvic lymph nodes, peritoneum and pleura. The bladder showed irradiation fibrosis but no signs of remnants of the tumour

Causes of Death

Thirty nine patients died during the observation period. The causes of death were well-established in 37 of these patients (see Tables 3 4 and 5)

It appears from Table 3 that death from carcinoma of the bladder was two and a half times as frequent among patients with primary carcinomas as among patients with primary papillomas. Among patients with primary papillomas, death due to thromboembolism was at least thrice as common as among patients with primary carcinoma. Data on the eleven patients who died from cancer of the bladder are given in Table 4 The interval between establishment of the diagnosis papilloma and the histologically established diagnosis of carcinoma varied from less than 6 months up to 15 years. In one patient (No. 32) clinical observations gave rise to the suspicion that malignancy might be in evidence he was immediately treated as a cancer patient and death was due to metastases.

Among the 37 patients who died during the observation period, 10 developed other tumours, which contributed to their death (see Table 5)

Changes in Dysplasia in Recurrence of Papillomas

Among 63 patients (papillomas grades 1 and 2) in whom the course was non-lethal, recurrence verified by histology was observed in twenty (32 per cent) Only one demonstrated persistently increased dysplasia (grades 1 → 2) Among 39 patients who died, recurrence was seen in 20 (51 per cent) Papilloma

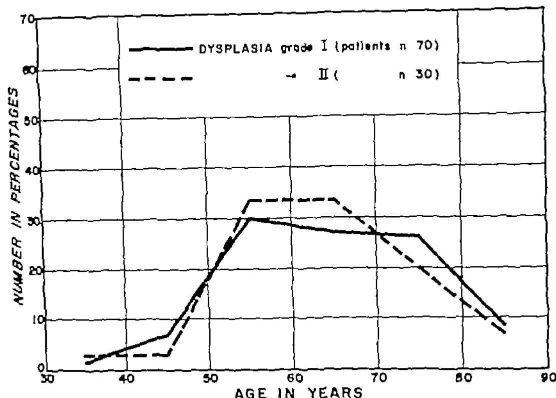


Fig. 3. Age distribution for patients with papillomas.

though unchanged dysplasia recurred in eight. In one (No. 31) a papilloma recurred and the grade of dysplasia increased. In the remaining eleven patients in the group of twenty carcinoma of the bladder developed (Table 4 and 5) In four of these (Nos. 25 53 59 and 88) the grade of dysplasia increased during the disease. In two patients, increased grade of dysplasia in the bladder carcinoma was demonstrated at autopsy (Nos. 53 and 120) Among the remaining five patients, the grade of dysplasia was unchanged

TABLE 3 *137 Main Causes of Death*

	Among 37 patients with papillomas	Among 87 patients with transitional cell carcinoma
Carcinoma of the bladder	11 (30 %)	67 (77 %)
Other cancer disease	10 (27 %)	9 (10 %)
Thrombo-embolic disease	17 (46 %)	11 (12 %)
Pneumonia	2*	2*
Complications involved in treatment	1	4
Suicide	1	
Perforated gastric ulcer		1
Accident	1	

Single cause of death.

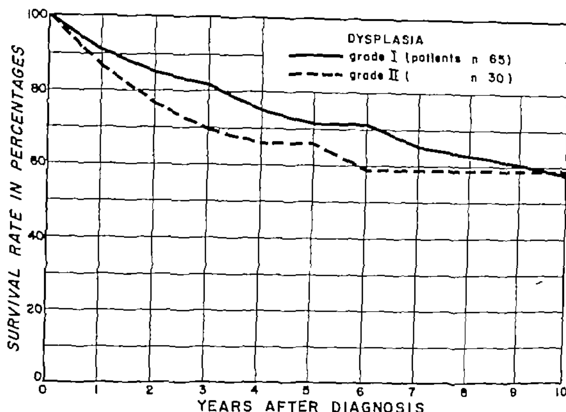


Fig 4 Cumulative survival curves for patients with papillomas.

in four (Nos. 77 120 133 and 203) and in one (No. 181) a change into squamous cell carcinoma was seen

Transitional Cell Carcinomas

This group comprises 112 patients. The age distribution appears from Table 2.

The sex distribution was male/female 87/25 Sixty-two tumours were papillomatous, fifty were non-papillomatous. The relation between age and grade of dysplasia at the time of the first diagnosis appears from Fig 5 A Kruskal Wallis test did not show any difference in the age distribution in relation to grade of dysplasia ($p > 0.10$)

The cumulative survival rates in relation to grade of dysplasia (grade 2 and 3) are demonstrated in Fig 6 The same survival rates applying to papillomatous and non-papillomatous carcinomas appears from Fig 7 Both calculations are based on findings in patients in whom, the bladder primarily was subjected to conservative therapy Both calculations show strong, significant differences in survival ($p < 0.0005$**) Corrections for differences in age distribution did not change these results.

Among patients with grade 2 tumours, the cumulative survival rates were higher if they were suffering from the papillomatous and not from the non-papillomatous type ($p < 0.01$**)

Only 11 patients in our series had carcinomas with dysplasia grade 1 This number is too small for a statistical calculation.

Staging

In 89 patients the grade of dysplasia and tumour staging (TNM system, UICC) were compared at the time of the first diagnosis. A correlation of these parameters appears from Table 6 The most severe grade of dys-

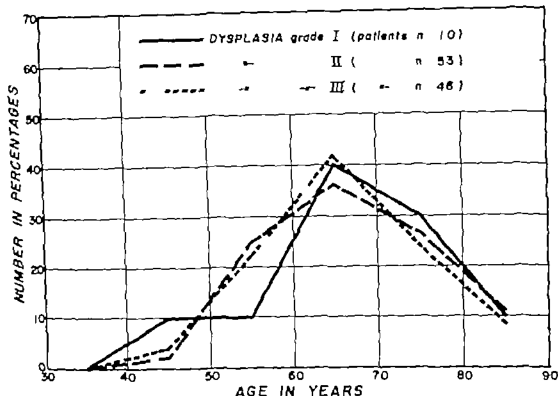


Fig 5 Age distribution for patients with transitional cell carcinoma.

plasia seemed to be almost evenly distributed among T + T and T + T_4. Dysplasia grades 1 and 2 were most common in the group with T_1 + T

Changes in Dysplasia at Recurrence of Carcinomas

Among 50 patients with carcinomas, local recurrence set in after supposed radical treatment. Forty-one of these recurrences were histologically verified. In 10 of the patients, the recurrence was in the form of papilloma, in 31 in the form of carcinoma.

The 10 *recurrences of papilloma* were found in patients who primarily had carcinomas with dysplasia grades 1 and 2. Seven of the recurrences were associated *with dysplasia* grade 1 even in 4 patients where the dysplasia in the original carcinoma was of grade 2. In the remaining 3 patients the recurrences were associated with dysplasia grade 2. Six of these 10 *patients in whom papilloma* recurred were still alive and without clinical signs of recurrence 2–12 years after the primary diagnosis had been established. Two succumbed to heart disease after 7 and 10 years, respectively and 2 died from carcinoma of the bladder within 2 years, i.e. less than one year after the recurrence appeared. Among the 31 patients in whom carcinoma recurred the grade of dysplasia in the recurrence was unchanged in 19 (61 per cent) seven in whom dysplasia was of grade 2 and 12 in whom it was of grade 3. In 9 patients, the grade of dysplasia had increased and in 3 with primary grade 3 carcinomas, the recurrence appeared as grade 2 carcinomas. Patients in whom carcinomas with dysplasia grade 2 recurred seemed to survive for longer periods of time than patients in whom carcinomas with grade 3 recurred.

TABLE 4 *Course of Disease in Eleven Patients*

Patient No	Primary diagnosis	Grade of dysplasia	Latent time in years	Carcinoma
25	Multiple papillomas.	1	14	Transitional cell gr. 3.
32	Multiple papillomas.	3	¾	Transitional cell gr 3.
44	Multiple papillomas.	1	½	No biopsy
53	Papilloma	1	3	Transitional cell gr 2.
59	Multiple papillomas.	1	3	Transitional cell gr. 3.
77	Papilloma.	2	1½	Transitional cell gr 2.
88	Multiple papillomas.	2	3	Transitional cell gr. 3.
120	Papilloma	1	½	Transitional cell gr 1.
135	Papilloma.	1	8	Transitional cell gr 1
181	Multiple papillomas.	1	½	Squamous cell.
203	Multiple papillomas.	1	15	Transitional cell gr. 1.

Changes in Papillomatous Non Papillomatous Type in Recurrent Carcinomas

Among 31 patients with histologically verified recurrence of carcinoma, the tumour type had changed in 12 according to the above mentioned parameter. Twenty two patients among the 31 had a primary papillomatous carcinoma. Nine of these (41 per cent) experienced a non papillomatous recurrence. In the remaining 13 patients with primary papillomatous carcinoma the tumour type had not changed in the recurrence. The survival time of the patients seemed to remain uninfluenced by changes in the tumour morphology most of these patients died within two years.

In 9 patients with primary non-papillomatous carcinomas, papillomatous carcinoma recurred in three. Eight of these 9 patients, including 2 of the 3 patients in whom papilloma recurred, died within one year after the carcinoma had recurred. The last patient in this group survived for 7 years after the first recurrence, although treatment of new recurrences included reresection of the bladder and later supervoltage radiotherapy. The cause of death was coronary thrombosis.

Post Mortem Examinations of Patients with Primary Bladder Carcinomas

The results obtained by post mortem examination of 66 bladders appears from Table 7. The final pathological result of papillomatous and non-papillomatous carcinomas seems to be identical. Ninety-four causes of death in a serie of 87 patients are listed in Table 3. As regards 8 patients with transitional carci-

Treatment	Survival time in years	Autopsy diagnosis	Clinical diagnosis
Supervoltage radiotherapy	>14	Transitional cell carc. gr 3 metastases to lymph nodes, peritoneum, uterus, adnexae, lungs.	
Supervoltage radiotherapy	1½	Metastases to peritoneum, pleura, gr 3	
Transurethral electrocoagul.	1½	None.	Bladder carc.
Cystectomia, urinary diversion.	3½	Metastases to pelvic lymph nodes, gr 3 reticulosarcoma.	
None.	3½	Coronary thrombosis. Prostatic carc., metastases to bone.	
Transurethral electrocoagul.	2	Transitional cell carc. gr 2, colloid breast carc., metastases.	
Transurethral electroresection.	4	Transitional cell carc. gr 3 metastases to uterus, parametrium, fossa Douglasi.	
Supervoltage radiotherapy	1¾	Transitional cell carc. gr 3 metastases to perivesical sites.	
Supervoltage radiotherapy	>14	None.	Pulmonary metastases.
Supervoltage radiotherapy	1	Ureterstenosis, pyonephrosis.	Uremia.
Transurethral electroresection	>15	None.	Carcinoma of the bladder metastases to bone.

noma, death was caused by another tumour (Table 8)

DISCUSSION

For more than a century the medical literature has comprised numerous works concerning the classification and grading of tumours of the bladder. *Rokitansky* (1861) seems to be the first who applied the term carcinoma to all villous bladder tumours while *Virchow* (1863 cit. Stenius) who misjudged these tumours used the term fibroma papillare and held that they were benign. *Albarran* (1892) distinguished between 3 types of epithelial tumours of the bladder. Allantoïdien, vésicule adulte et proliférations atypiques. He emphasized that papillomas should not be considered benign, and that difficulties were involved in conclusions about the prognosis drawn on the bases of the macroscopic and microscopic structure of the tumours. This point of view has been confirmed in our day (*Greene et al* 1973). It is generally accepted by authors that transitional cell tumours are malignant as soon as there is atypia of the urothelium (*Zuckerkandl* 1909 *Broders* 1922, *Pugh* 1957 *Mostofi* 1960 1968 *Lerman et al* 1970). Only few have emphasized that patients with papillomas but absence of dysplasia may survive for just as long a time as the rest of the population (*Lerman et al.* 1970) *Schaldemose* (1904) found that some tumours of the bladder regardless of their clinical behaviour showed cell atypia, while others did not. *Stenius* (1925) classified bladder tumours as benign and malignant papillomas, papillomatous and solid carcinomas. He measured nuclei and nucleoli in the tu-

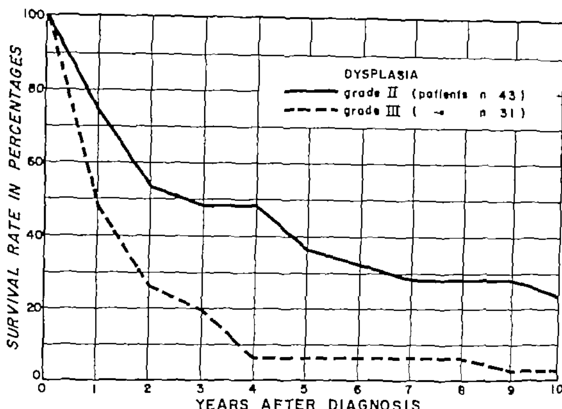


Fig 6 Cumulative survival curves for patients with transitional cell carcinoma. Conservative surgery

mours and found that they were larger than those in the normal urothelium, increasing in size with malignancy *Franksson* (1950) used 7 grades according to the cell atypia Grade *one was benign, grades 4–7 were malignant* with infiltration of the stroma. However this grading emphasized primarily the atypia of the urothelium, secondly the relation between the diseased urothelium and stroma. *Berg kvist et al.* (1965) used the same principle in their classification of tumours of the bladder according to cellular pattern and found a good correlation between the degree of cellular deviation and survival. They considered grade 0 and grade 1 tumours benign although a grade 4 recurrence developed in one patient with a grade 1 tumour 5 years after removal of the primary tumour Invasion of the tumour however implies a poorer prognosis. The same principle has also been applied by *Sorensen et al.* (1969) who excluded grade 0 A correlation between histological grade and clinical stage was demonstrated. The same applied to histological grade and survival. *Brons et al.* (1972) used also this histological grading They found correlation between stage and grade and demonstrated that the prognosis was most favourable in patients in whom grades were low and stages not advanced The five-year survival was 21 4 per cent in their series of patients, irrespective of the treatment the trial covered a period of 15 years. *Schroeder et al* (1973) applied Mostofi s and WHO's grading to bladder carcinomas from 145 patients. The five year survival was 33 per cent, 20 per cent and 19 per cent in cases of dysplasia grade 1 grade 2, and grade 3 respectively These figures are based on the total number of patients without regard to papillomatous, non-papillomatous structure of the carcinomas.

According to the classical histological criteria of malignancy we have classified the

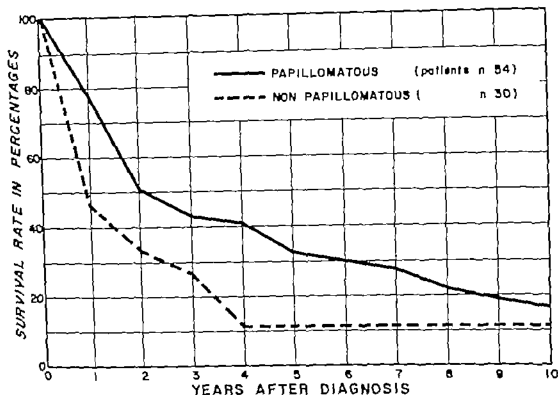


Fig. 7 Cumulative survival curves for patients with transitional cell carcinoma. Conservative surgery

bladder tumours observed in this trial in two main groups. Tumours without (papillomas) and tumours with destructive invasion, and these are subdivided into papillomatous and non-papillomatous carcinomas. The degree of dysplasia of the epithelium in all these tumours was determined. Our results are in support of the value of this procedure which seems to be useful and practical in the routine. It is neither too complicated nor too sophisticated to be comprehended by pathologists.

In our series of patients with papilloma we have not had opportunity to follow patients in whom dysplasia was of grade 0 for any length of time.

Papillomas associated with dysplasia grades 1 and 2 were not related to age. The 5-year survival rate in cases of papilloma with dysplasia grades 1 and 2 seemed to be one and the same. In two patients with papillomas associated with dysplasia grade 3 the latter behaved like papillomatous carcinoma and the prognoses were equally poor. In this connection it should be remembered that the *grade of malignancy may vary in different* parts of the tumours (*Frankson* 1950). This may also explain the changes in dysplasia which have been observed in some patients, although it is generally believed that recurrences tend to be of a higher grade of malignancy. In our series, however, dysplasia was rarely seen to change in patients in whom papilloma recurred whether the course was lethal or non-lethal. On the other hand an increase in dysplasia seemed to be more common among patients with papilloma in whom the recurrence was a carcinoma.

According to our opinion it seems reasonable to maintain *groups with papillomas* associated with dysplasia grades 1 and 2 on the consideration that the prognosis is more favourable than that of patients with carcinoma. In cases of papillomas with dysplasia

TABLE 5 *Ten Patients with Primary Bladder Papillomas who Developed Extravesical Malignant Tumours*

Patient No.	Diagnosis	Grade of dysplasia	Survival in years	Tumour of the bladder at time of death	Other tumours
31	Multiple papillomas.	1	4	None.	Adrenal pheochromocytoma.
38	Papilloma.	1	1½	None.	Bronchial carcinoma with metastases.
53	Multiple papillomas.	1	3½	None	Reticulosarcoma retroperitoneal, mediastinal.
59	Multiple papillomas.	1	3½	Transitional cell carc. dyspl. gr 3	Prostatic carcinoma, metastases to bone.
71	Papilloma.	2	1	None.	Bronchial carcinoma.
77	Papilloma.	2	2	Transitional cell carc. dyspl. gr 2.	Colloid carcinoma of the breast with metastases.
115	Papilloma.	2	4½	? (no autopsy)	Bronchial carcinoma.
130	Papilloma.	1	9	None.	Hypernephroma with metastases.
152	Multiple papillomas.	1	1	? (no autopsy)	Prostatic carcinoma with metastases
231	Papilloma.	1	3	None.	Cerebral astrocytoma gr IV

TABLE 6 *Comparison between Grade of Dysplasia and Tumour Staging at the Time when the First Diagnosis was established in 89 Patients*

Dysplasia	T + T	T + T	Total
I + II n	40	12	52
%	77	23	100%
III n	21	16	37
%	57	43	100%
Total n	61	28	89

grade 3 the prognosis is by far less favourable. It is of interest to observe that trombo-embolic diseases are the most common cause of death in the group of patients with papilloma. Carcinoma of the bladder developed in about 30 per cent of the patients in this group. Other tumours developed in 20 per cent of these patients, *in other words, a per* centage equally that in the general population. It is remarkable that the non-papillo-

TABLE 7 *Post Mortem Examinations of Carcinomas of the Bladder*

	Papillomatous 34 autopsies		Non-papillomatous 32 autopsies	
	n	%	n	%
Tumour remnant in the bladder	28	82	25	78
Tumour penetration of bladder wall	20	59	18	56
Metastases	18	53	17	53

TABLE 8. *Eight Patients with Transitional Cell Carcinoma whose Death was Caused by Another Cancer*

Patient No.	Extra vesical tumour	Metastases	Tumour remnant in bladder
15	Bronchial carcinoma	—	+
27	Prostatic adenocarc.	—	+
68	Prostatic adenocarc.	+ (bladder)	+
118	Hypernephroma	+	—
156	Prostatic adenocarc.	+ (bladder)	+
202	Bronchial carcinoma	—	—
121	Rectal adenocarc.	—	+
227	Prostatic adenocarc.	—	+

matous carcinomas occurred in the elderly patients in our series, while the papillomatous carcinomas were found to develop in patients in the younger age groups. Why some carcinomas become papillomatous while others become non-papillomatous is still unknown. It could be an expression of some unknown defence in the patient, a postulation which is supported by the fact that the rate of survival estimated in the traditional way is much higher among patients with papillomatous carcinoma than among those with non-papillomatous (*Lund & Lundwall* 1958, *WHO*). On the other hand, we have not demonstrated a relation between grade of dysplasia and age. The higher rate of survival after dysplasia grade 2 as compared with grade 3 in a group of almost uniformly treated patients is in accordance with the findings obtained by other investigators and indicates that the grade of dysplasia is an expression of malignancy of tumours.

Owing to the modest number of patients in our series it has been divided into two groups according to the T-staging T + T and T + T. It was rather surprising that dysplasia grade 3 was found to be almost evenly distributed over the two groups. Dysplasia grades 1 + 2 was most common in T + T. Table 7 demonstrates that the final dissemination of the two groups of carcinoma apparently is the same, whatever the carcinoma form, although the duration of the disease seems to differ.

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The statistical methods applied are the * Kruskal-Wallis test and the ** survivorship function.

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UROTHELIAL CHANGES OF THE RENAL PAPILLAE IN SPRAGUE-DAWLEY RATS INDUCED BY LONG TERM FEEDING OF PHENACETIN

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Johansson, S. & Angervall, L. Urothelial changes of the renal papillae in Sprague Dawley rats induced by long term feeding of phenacetin. Acta path. microbiol. scand. Sect. A, 84 375-383 1976.

Thirty female Sprague Dawley rats were fed 0.535 per cent phenacetin in the diet for up to 110 weeks. Twenty-six of these rats developed urothelial hyperplasia, partly papillary of the renal papillae. Twenty-eight rats showed dilatation of the vasa recta frequently associated with thrombus formation and calcification. One phenacetin fed rat had epithelial hyperplasia associated with chronic pyelitis. In 2 of the 30 control rats urothelial hyperplasia was found to be associated with chronic pyelitis. The hyperplastic urothelial changes and vascular changes were often, but not always present simultaneously. One control rat developed a mammary carcinoma, as compared with 5 rats in the phenacetin group. Four phenacetin fed rats developed carcinoma of the ear duct. The results of the present investigation provide evidence that phenacetin can induce proliferative lesions of the urothelium of the rat renal pelvis with weak carcinogenic activity in the ear duct and mammary glands.

Key words Urothelial changes phenacetin rats.

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In 1953 *Spühler* & *Zollinger* described a high incidence of interstitial nephritis and renal papillary necrosis in patients whose intake of analgesic compounds containing phenacetin was heavy. Since then numerous reports particularly from the Scandinavian countries, Switzerland, and Australia have confirmed that observation (e g *Schourup* 1956 (*loor* 1961 *Bengtsson* 1962, *Kincaid Smith* 1969) In addition, an association between high intake of phenacetin-containing drugs and development of renal pelvic tumours has been demonstrated (*Hultengren et al.* 1965 *Bengtsson et al* 1968, *Angervall et al* 1969 *Johansson et al* 1974)

It has been difficult to induce similar kidney lesions in experimental animals. A successful attempt at inducing renal papillary necrosis by phenacetin alone was reported by *Clausen* (1962, 1964) Three out of 25 rabbits fed phenacetin developed renal papillary necrosis. Using very large doses of phenacetin, *Fordham et al.* (1965) induced papillary necrosis in 3 out of 39 rats over a period of 4 weeks. *Nanra et al* (1971) obtained similar results and by subjecting the rats to daily dehydration a higher incidence of papillary

necrosis (37.5 per cent over 8–20 weeks) was induced. In rats fed phenacetin for up to 41 weeks, the renal concentration ability was impaired but recovered on withdrawal of the drug (*Angervall et al.* 1964 and 1968). Experimental studies of phenacetin have focused on inducing renal papillary necrosis and/or interstitial nephritis. Only in the study by *Schmähl & Reuter (1954)* where rats were fed up to 22 grams of phenacetin over a period of 150–600 days, was the purpose to induce cancer. No evidence of urothelial or other tumours were reported. In a recently published study (*Johansson & Angervall* 1976) 40 rats were fed 0.535 per cent phenacetin in the diet for up to 86 weeks. The total amount of ingested phenacetin was approximately 55 grams. In 6 rats fed phenacetin, severe hyperplasia of the urothelium covering the renal papillae was found and 15 had mild to moderate hyperplasia as compared with the control rats. These findings provided the basis for a second experiment aiming at inducing tumours of the renal pelvis by prolonging the time interval during which they were exposed to phenacetin. The results from this experiment are presented in this paper.

MATERIAL AND METHODS

Experimental design. Sixty 6-week-old female Sprague-Dawley rats supplied by Anticimex AB, Stockholm were used. The initial weight of all the rats was approximately 95 grams. The rats were divided into 2 groups. Group I (30 rats) received pellets containing 0.535 per cent phenacetin. Group II (30 rats) received pellets without phenacetin. Both diets were supplied by Astra Ewos AB, Södertälje, Sweden.

The rats had free access to food and tap water. The rats were kept under constant temperature (23–24 C), constant humidity (60 per cent relative humidity) and light (12 hours light and 12 hours darkness) throughout the whole experiment. The rats were kept in plastic cages with 6 rats in each cage. The rats were weighed and the food consumption was determined periodically during the experiment.

Seventeen rats fed phenacetin and 18 control rats were sacrificed after 110 weeks. The other rats died or were sacrificed earlier during the experiment due to the development of malignant tumours and/or kidney disease. The average survival time was 94 weeks in the case of rats fed phenacetin and 102 weeks in the case of the controls.

Breast tumours developed in all long-surviving rats except 3 rats fed phenacetin and 3 control rats. The tumours were excised under ether anaesthesia in order to prevent complications such as ulceration, bleeding and infection.

Examination of organs. A post mortem examination was performed on all rats, and all grossly abnormal organs were submitted to histological examination. Both kidneys were removed, weighed, and fixed in 10 per cent buffered formalin. The bladder was inflated and fixed in 10 per cent buffered formalin. Multiple transverse sections were made from the kidneys and were embedded in paraffin. From these, 5 μm sections were stained with haematoxylin-eosin and haematoxylin-van Gieson. Van Kossa stain was also performed on paraffin sections. Pieces of the kidney medulla was embedded in metachrylate and from the blocks 1 μm sections were stained with haematoxylin-eosin, haematoxylin-van Gieson, and PAS-staining (*McManus 1946*).

The vascular changes of the renal papillae were classified as mild to moderate or severe. The epithelial lesions were classified as mild, moderate, or severe.

Statistics. The statistical calculations were performed according to Fisher's non-parametric permutation test and trend in a contingency table which is a variation of that test (*Odén & Wedel* 1975).

RESULTS

The dietary intake was 25 per cent lower among the phenacetin rats throughout the experiment. The average body weight of the rats surviving 110 weeks was 312 grams (S.E. ± 10) for the phenacetin fed rats (n = 17) and 406 grams (S.E. ± 22) for the control rats (n = 18). The difference is statistically significant ($p<0.01$). The combined kidney weight was 2.92 grams (S.E. ± 0.12) for the phenacetin rats and 3.74 (S.E. ± 0.41) for the control rats. The difference is not statistically significant ($p>0.06$).

Histological Types of Kidney Lesions

I. *Epithelial* lesions. Hyperplasia of the epithelium covering the renal papillae was found in 26 rats fed phenacetin and in 5 control rats (Table 1). The normal rat papillae

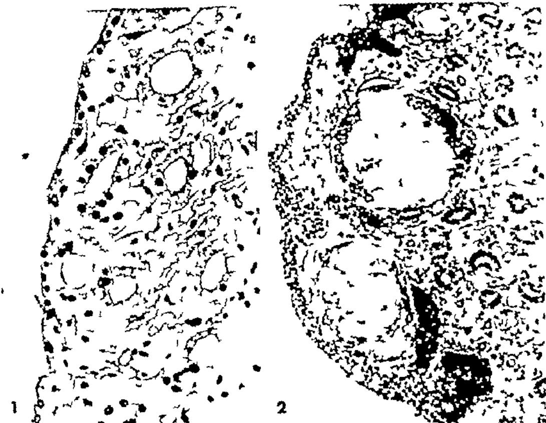


Fig. 1. Normal epithelial lining of the renal papillae from a 110 weeks old control rat. Metachrylate embedded 1 µm section, H & E, × 234.

Fig. 2. Dilated vasa recta with thrombus formation and mild to moderate urothelial hyperplasia in a Sprague-Dawley rat fed phenacetin for 107 weeks. Metachrylate embedded 1 µm section, H & E, × 120.

is covered with one single layer of cuboidal epithelium (Fig. 1) even in ageing rats (*Lalich et al.* 1974). The epithelial hyperplasia was found to be associated with the vascular changes and/or calcification within the organized thrombi or interstitially (Fig. 2, 3, 4). The hyperplasia was usually focal and flat, occasionally papillary (Fig. 5, 6). Sixteen rats fed phenacetin had severe epithelial bilateral hyperplasia and 5 moderate (Fig. 7). In two cases, nuclear atypia and mitoses were found (Fig. 8). In one rat fed phenacetin and presenting severe epithelial hyperplasia it was also associated with unilateral chronic pyelitis with squamous metaplasia and cornification. In two control rats, moderate to severe hyperplasia was associated with chronic pyelitis (Table 1). The difference in degree and frequency of the epithelial changes in rats fed phenacetin and control rats is statistically significant ($p < 0.01$).

II. *Vascular* lesions. Vascular lesions were found in 28 rats fed phenacetin and in 8 control rats (Table 1). The branches of the vasa recta extending close to the surface of the papillae frequently were dilated and congested with thrombus formation (Fig. 2). The thrombi often became organized and calcified (Fig. 3). In one rat fed phenacetin there was also coexisting chronic pyelitis associated with severe vascular changes. In two control rats severe vascular changes were associated with chronic pyelitis (Table 1). The difference in the degree and frequency of vascular changes





Fig 3 Severe vascular changes of the vasa recta with thrombus formation and calcification in association with the severe diffuse urothelial hyperplasia in a Sprague-Dawley rat fed phenacetin for 110 weeks. Metachrylate embedded 1 μm section, H & E, × 48.

Fig 4 Severe focal urothelial hyperplasia and calcification of the renal papillae in a Sprague-Dawley rat fed phenacetin for 110 weeks. Metachrylate embedded 1 μm section, H & E, × 120

TABLE 1 *Degree and Frequency of Histological Changes in the Renal Papillae of Female Sprague-Dawley Rats after Long-Term Treatment with Phenacetin*

Histological changes	Rats fed phenacetin (n = 30)	Controls (n = 30)
Mild urothelial hyperplasia	5	3
Moderate urothelial hyperplasia	5	1
Severe urothelial hyperplasia	16	1
No urothelial hyperplasia	4	25
Mild to moderate vascular changes	8	6
Severe vascular changes	20	2
No vascular changes	2	22

in rats fed phenacetin and control rats is statistically significant ($p<0.01$)

III. *Renal cortical changes* In six control rats and in 1 rat fed phenacetin deterioration was rapid and they presented large, pale granulated kidneys at autopsy Histologically the kidneys showed marked dilatation of the tubules and the same applies to the collecting ducts and Bowman's space. Most of the





Fig 5 Severe papillary hyperplasia of the renal papillae in Sprague-Dawley rat fed phenacetin for 110 weeks. H & E, × 75

Fig 6 Diffuse severe partly papillary hyperplasia of the epithelium of the renal papillae in a Sprague-Dawley rat fed phenacetin for 110 weeks. Metacrylate embedded 1 μm section, H & E, × 75

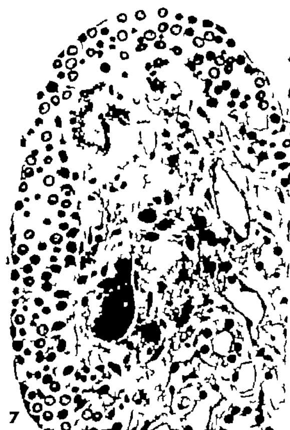
TABLE 2. *Histological Type and Frequency of Malignant Tumours in Female Sprague-Dawley Rats after Long-Term Treatment with Phenacetin*

Type of tumour	Rats fed phenacetin (n = 30)	Controls (n = 30)
Adenocarcinoma of the breast	5	1
Squamous cell carcinoma of the ear duct	4	0
Adenocarcinoma of the ovary	1	1
Adenocarcinoma of the pancreas	0	1
Squamous cell carcinoma of the oral cavity	0	1

glomeruli showed focal glomerulitis and/or hyalinization. There was marked fibrosis with focal interstitial lymphocytic infiltration. Hyalin casts were frequently accompanied by a large number of polymorphnuclear leucocytes in the dilated tubules, collecting ducts and Bowman's space.

The urinary bladder epithelium was mildly hyperplastic in 2 rats fed phenacetin, one of which had moderate chronic cystitis.

Other neoplasms Seventeen rats fed phenacetin and 25 control rats developed fibroadenomas of the breast during the experiment. The first tumours appeared after 8 months of the experiment. In 16/17 rats



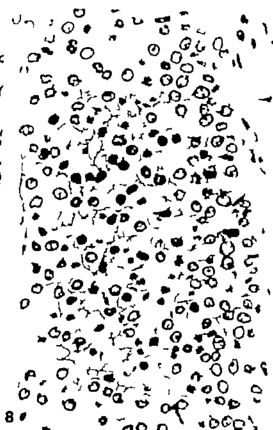


Fig 7 Mild to moderate epithelial hyperplasia of the renal papillae in a rat fed phenacetin for 110 weeks. Metachrylate embedded 1 µm section, H & E, × 300

Fig 8 Diffuse severe hyperplasia of the epithelium of the renal papillae in a Sprague-Dawley rat fed phenacetin for 110 weeks. N te the mitosis. Metachrylate embedded 1 µm section, H & E, × 300

fed phenacetin and 21/25 control rats the tumours recurred after the primary excision and were removed one or several times, up to 11 Five rats fed phenacetin and 1 control rat developed mammary adenocarcinomas, one of which metastasized (phenacetin rat) to the lungs. The other tumours infiltrated into the intercostal muscles they were inoperable and resulted in the death of the rats. Four rats fed phenacetin developed ear duct tumours, histologically squamous cell carcinomas, one of which metastasized to the lungs (Table 2)

DISCUSSION

The rats fed phenacetin had retarded growth in comparison with the control rats, the mean body weight being almost 100 grams lower per rat at the end of the experiment. This may be explained by the lower dietary intake as well as increased motor activity and restlessness. This was observed in a previous study by *Angervall & Bengtsson* (1968) Human analgesic abusers without renal insufficiency have also been found to have lower body weight than non abusers (*Bengtsson* 1966)

The difference in the degree and frequency of the urothelial hyperplasia in the rats fed phenacetin and the control rats is statistically significant. The results are in accordance with those obtained in an earlier experiment (*Johansson & Angervall* 1976) but the frequency and degree of the urothelial lesions is increased in the present experiment. This

is probably due to a longer exposure to phenacetin. However no rats exhibited infiltrating or metastasizing urothelial tumours.

Twenty-eight out of 30 rats fed phenacetin had dilated vasa recta and, in 20 rats, these vascular changes were classified as severe. The difference in frequency and degree of the vascular changes between the rats fed phenacetin and control rats is statistically significant ($p<0.01$). In the rats with severe vascular changes, thrombus formation with calcification was frequently found. The changes of the vasa recta of the renal papillae were frequently but not always, associated with urothelial hyperplasia of the papillae. *Kincaid-Smith et al.* (1968) demonstrated changes in the vasa recta at the corticomedullary junction by way of electron microscopy as well as light microscopy after feeding analgesic compounds containing phenacetin. The changes consisted in a reduced number of vessels and decreased diameter of the lumen of the vasa recta combined with perivascular fibrosis. Also dilated vessels were noticed. Hence it seems probable that the vascular changes of the renal papillae observed in the present study were induced by phenacetin and developed independently of the urothelial hyperplasia.

Lalich et al. (1974) demonstrated dilatation of the vasa recta in uninephrectomized rats after treatment with monosodium glutamate or excess sodium chloride in the diet. These vascular changes were associated with epithelial hyperplasia but since multiple factors were employed in the experiment no conclusions were drawn about the cause of the dilatation.

The renal cortical changes were observed in the present study similar to those reported to occur in ageing rats (*Saxton & Kimball* 1941, *Kennedy* 1957, *Simms & Berg* 1957). In the study by *Simms & Berg* (1957) severe cortical changes were found in almost 30 per cent of the rats after two years of life. Our finding of a lower incidence among the rats fed phenacetin may at least partly be explained by the shorter survival of these rats.

Thus, the present investigation has shown that it is possible to induce urothelial papillary hyperplasia of the renal papillae in rats after long term feeding of phenacetin. However the exact mechanism in the development of the urothelial hyperplasia is unclear. N-hydroxy phenacetin has been described as a metabolite of phenacetin in cats, dogs and man (*Klutch et al.* 1966). The identification of a condensation product, i.e. azoxy-4-ethoxy-benzene of N-hydroxy phenacetin or N-hydroxy-4-phenetidine in the urine of phenacetin treated rats (*Nery* 1971 a) may indicate, according to *Nery* (1971 c), a concentration of the N-hydroxylated metabolites in the kidney as has been described for 4-acetamino-phenol (*Blumle & Goldberg* 1968) which is the major metabolite of phenacetin in all species investigated. It has also been found that (ethyl-C^{14})-phenacetin results in a greater binding of radioactive label to liver nucleic acids as compared with (acetyl-C^{14}) phenacetin (*Nery* 1971 b). N-hydroxylation and N-hydroxyesterification are considered to be the first and second activation steps, respectively, in the carcinogenesis of aromatic amines and amides (*Miller & Miller* 1969). These considerations strongly support that phenacetin abuse represents a highly probable carcinogenic hazard (*Nery* 1971 c).

In the present study small areas of focal calcification and necrosis were seen in 10 rats, but frank renal papillary necroses were not found. In a previous study (*Johansson et al.* 1974) 57 out of 62 patients in whom heavy intake of phenacetin-containing analgesics was combined with the development of urothelial renal pelvic tumours had papillary necrosis. In 3 patients, papillary necrosis could be excluded. Hence, renal papillary necrosis is not a prerequisite for the development of urothelial renal pelvic tumours after ingestion of phenacetin.

A study of the influence of phenacetin and of the strong urinary tract carcinogen N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) on the cellular and humoral immune status in male Fischer rats was recently performed (*Johansson et al.*, to be published). Neither were immunosuppressive in contrast

to many chemical carcinogens, particularly the carcinogenic polycyclic hydrocarbons (*Baldwin* 1973) This would be in accord with the more recent suggestion that the theory of immunosurveillance may not be applicable to the aetiology of non-lymphomatous neoplasms (*Schwartz* 1975) Thus, it appears from the present study that the development of urothelial renal pelvic papillary hyperplasia in rats fed phenacetin was due, at least partly to a direct action of phenacetin metabolites on the urothelium. In addition, the high frequency of mammary and ear duct carcinomas among the rats fed phenacetin may imply a more general carcinogenic effect of phenacetin.

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A SYNERGISTIC EFFECT OF OESTRADIOL AND PROLACTIN INFLUENCING THE INCIDENCE OF 3-METHYLCHOLANTHRENE INDUCED CERVICAL CARCINOMAS IN MICE

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Forsberg, J.-G. & Stray Breistein, L. A synergistic effect of oestradiol and prolactin influencing the incidence of 3-methylcholanthrene induced cervical carcinomas in mice. Acta path. microbiol. scand. Sect. A, 84 384–390 1976

Castrated NMRI mice were laparotomized and a thread impregnated with beeswax methylcholanthrene was inserted into the uterine cervix. Beginning on the day of operation and for a further 5 days the animals were injected with oestradiol, prolactin, oestradiol-prolactin, oestradiol-prolactin-progesterone, or the solvents for the hormones only. One group of animals were injected with oestradiol-prolactin for 6 days and later with progesterone every third day until death. The animals were killed one or 4 weeks after the operation. Among the one-week animals the number of cervices presenting epithelial downgrowths ("buds") into the stroma was higher after treatment with a combination of oestradiol and prolactin than after treatment with each hormone separately or among the controls. Four weeks after operation, the incidence of squamous cervical carcinomas was seen to be significantly higher among animals injected with both oestradiol and prolactin than in controls or in those injected with oestradiol or prolactin alone. Progesterone had no definite effect on the oestradiol-prolactin induced incidence. The mechanism behind the synergistic effect of prolactin and oestradiol is discussed.

Key words: mouse cervical carcinomas; prolactin; oestradiol; progesterone; incidence of carcinomas.

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Hormonal factors are generally not considered to be *of primary importance in human uterine cervical carcinogenesis* (3). Reports on the effect of hormones on induction and growth of experimental cervical carcinoma have been conflicting. Oestrogen and castration are factors which have been described as both hastening and retarding cervical carcinogenesis, the same holds true of gestagens (5, 8, 18, 20). As the cervical epithelium physiologically is a target tissue for sex steroids, these conflicting reports may be somewhat surprising. Maybe they are real, *reflecting differences between strains*, type of carcinogen used, etc., but they may also reflect insufficiencies in the experimental test model. As regards the human disease, factors e.g. regulating the transition from carcinoma *in situ* to invasive cancer are unknown (2).

Hormonal factors should not a priori be disregarded merely because it has not been possible to demonstrate an effect of these.

We have earlier compared the 3-methyl-

cholanthrene (MCA) induced cervical carcinogenesis in normal and neonatally oestrogenized NMRI mice (5). These two groups of animals are different in their endogenous hormonal balance, the oestrogenized animals having an ovary-dependent persistent vaginal cornification (1, 4). The initial incidence of invasive cervical lesions was higher in the oestrogenized animals than in normal animals, but the persistent production of oestrogen seemed to retard cancer growth (5). Taking different factors into consideration, our interest was directed to prolactin because serum prolactin levels are higher in neonatally oestrogenized animals than in control animals (19) and because prolactin has been shown to exert an effect on the vaginal epithelium (11, 17, 23).

In further studies, explants of cervical carcinomas from normal and oestrogenized mice were cultured as organ cultures in the presence of prolactin and ^{3}H thymidine was added during the final hour of incubation (6). Prolactin stimulated the thymidine incorporation into carcinomas from control animals and had probably some effect on tumours from oestrogenized animals.

In the present paper, an account is given of the results obtained in an *in vivo* study, the object of which was to investigate the effect of prolactin, oestradiol and progesterone on MCA induced cervical carcinogenesis in normal, castrated mice.

MATERIALS AND METHODS

Animals

All animals belonged to a closed, randombred NMRI strain; they were fed standard pellet diet and given water *ad libitum*. Pregnant females gave birth to their litters in separate cages. As the animals comprised in this series were to be used as control animals in other experiments, they were all given 0,025 ml olive oil daily for the first five days after birth.

Operation.—At the age of 6–9 weeks the females were castrated and one week later laparotomized. A cotton thread impregnated with a mixture of 3-methylcholanthrene (MCA, Sigma Chemical Co.) and beeswax (1:3 by weight) was inserted into the uterine cervix by the method described earlier (5).

Injections

After insertion of the MCA thread, the animals were divided into a "short-term" and a "long-term" experimental group.

Each group included sub-groups where the animals received different hormonal treatment. In one group, the animals were injected with 5 μg ovin prolactin (50IU/ml, Sigma Chemical Co) in 0.1 ml Parker 199 and 0.05 ml ethanol-saline (1:10) at different sites; animals in a second group received 5 μg oestradiol 17β (Sigma Chemical Co) in 0.05 ml ethanol-saline (the suspension was carefully shaken before use) and 0.1 ml Parker 199; a third group was injected with 5 μg prolactin and 5 μg oestradiol dissolved as above. Animals in the control group were injected with 0.1 ml Parker 199 and 0.05 ml ethanol-saline. The injections were started on the day of operation and were repeated daily for 6 days. In the "short-term" group, the animals were killed the day after the last injection; in the "long-term" group the animals were killed 22 days after the last injection.

The "long-term" experiments included a further three sub-groups which had no counterparts in the "short-term" experiments. In one of the sub-groups, the animals were injected for 6 days with oestradiol, prolactin (amount as above) and progesterone (1 mg in olive oil). Another sub-group consisted of animals injected with oestradiol and prolactin for 6 days; subsequently progesterone (1 mg in olive oil) was injected every third day until the animals were killed. Finally, the third sub-group served as control for the last-mentioned group; the animals were injected according to the same time schedule, using vehicles for the hormones only.

Histological Technique

After killing of the animals (cervical dislocation), the uterine cervix and fornicate region of the vagina was dissected out, fixed in Bouin's fluid, embedded in paraffin and serially cut at a section thickness of 7 microns. The sections were stained in haematoxylin and eosin. No others but cervices where the thread was definitely in place at dissection were studied.

RESULTS

"Short-term" Experiments

All the 8 control preparations studied one week after insertion of the MCA thread had a stratified squamous cervical epithelium. Regions presenting a mild epithelial atypia were interspersed within this normal epithelium. The nuclei of the basal layer and

in varying heights of the epithelium were spindle-shaped and showed hyperchromasia. Scattered micro-abscesses and invasion of leucocytes were seen in the epithelium. In one of the eight preparations, a few scattered epithelial "buds" were seen to penetrate into the stroma (Fig 1) These buds took their origin either from the normal-looking epithelium or from regions with mild atypia.

Two out of 8 cervices from oestradiol injected animals had buds similar to those described above. While in most cases, the buds had an intact basal membrane, its existence was questionable in some regions Moreover parallel to the occurrence of buds there were signs of a direct stromal invasion of cells from the basal epithelial cell layer

Nine animals received only injections of prolactin. Two out of the nine cervices demonstrated the occurrence of buds, in some cases with a questionable basal membrane and presumed invasion.

In strong contrast to the groups earlier described, buds were demonstrable in 6 out of 9 cervices from animals injected with both prolactin and oestradiol. The impression was that buds were more numerous and larger in this experimental group than in those described above. Several buds showed signs of overt invasion and also small invasive lesions, seemingly not related to buds, occurred (Fig 2)



Fig 1 Control animal, one week after insertion of the MCA thread. Buds penetrating from the cervical epithelial lining into the stroma. Magnification 220 ×

TABLE 1 *Incidence of Experimental Cervical Carcinomas in the Mouse* "Long term" experiments

	Influence of hormonal treatment		
...tment groups	No. of animals with carcinomas	No. of animals without carcinomas	Incidence per cent
... E_2 + P	8	2	80
... E_2 + P + Pr*	8	4	67
3 E_2 + P + Pr‖	7	7	50
4 E_2	2	11	15
5 P	3	12	20
6 C1†	4	10	29
7 C2‡	2	9	18

E_2 oestradiol-17β P prolactin Pr progesterone C controls.
Progesterone injected together with oestradiol prolactin for 6 days
‖ Progesterone every third day after 6-day treatment with oestradiol-prolactin.
† Controls for groups 1 and 4–5
‡ Controls for E + P + Pr‖



Fig. 2



Fig. 3



Fig. 4

Figs. 2–4 Prolactin and oestradiol injected animals. Fig. 2 demonstrates a small invasive lesion in an animal killed one week after insertion of the MCA thread. Figs. 3 and 4 show lesions in animals killed 4 weeks after insertion of the thread. Fig. 3 demonstrates the histological appearance of a squamous carcinoma. Fig. 4 is a low-power magnification demonstrating the penetration of the whole cervical wall with carcinomatous tissue. Magnification Fig. 2 and 4 220 × Fig. 3 42 ×

Long-term" Experiments

The control group C1 (see Table 1) consisted of 14 animals. Regions with atypical epithelium of the type described above were interspersed in regions with a normally stratified epithelium. In one of the preparations, a lesion suggestive of adenocarcinoma in the uppermost part of the cervix was noted in a further 3 animals early squamous carcinomas were seen.

In 7 out of the 13 animals injected with oestradiol only and in 3 out of 15 prolactin injected animals, small invasive lesions occurred in the cervical canal.

Again, in strong contrast to the previously mentioned groups, 8 of the 10 animals injected with prolactin and oestradiol had cervical carcinomas which, in one case invaded the cervical wall to the mesothelial surface (Fig. 3 4). In the remaining 7 cases, the carcinomas were of varying size, but involving larger parts of the cervical wall than in any other experimental groups belonging to Long-term" experiments. With the excep-

tion of one animal in which co-existence of an adenocarcinoma and a squamous carcinoma was observed, all the carcinomas were of a moderately well-differentiated squamous type.

As seen from Table 1 the incidence of carcinomas was slightly higher among the animals given oestradiol and prolactin than among those receiving additional progesterone, either administered only for the first 6 days, or in the form of repeated injections every third day until death. A consistent trait of the latter treatment was the pronounced mucification of the carcinomas as well as a strong leucocyte infiltration (Fig. 5) Otherwise there were no obvious differences between animals injected with oestradiol prolactin alone or progesterone in addition as regards the localization or the involvement of the cervical wall.

A chi-square analysis (after normal approximation of the binomial distribution, $\chi^2 = \Sigma t$) showed a statistically significant heterogeneity between treatment groups 1–7 (Table 1 $\chi^2 = 12.904$ for 6 d.f. $0.05 > p > 0.01$) There was no heterogeneity between treatment groups 1–3 or 4–7 When groups 1–3 were treated as one material and groups 4–7 as another the difference between these two materials was highly significant ($\chi^2 = 16.895$ 1 d.f., $p < 0.001$)

DISCUSSION

The epithelial "buds" which in the "short term" experiments were found to penetrate into the stroma and to occur both in experimental and control groups are also seen in cases of e.g 3 4-benzpyrene induced cervical carcinogenesis (21) In other studies using MCA, the buds have been interpreted as very early invasive lesions (5 9) Since transient downgrowths of a similar type also were observed if an intracervical thread impregnated with beeswax only was used (5) the term "bud" is preferable as long as the developmental fate of the downgrowth is unknown. No doubt, several buds are the starting point for invasive carcinomas, but they may also be



Fig 5 Detail of a squamous carcinoma in an animal injected with oestradiol-prolactin for 6 days and thereafter with progesterone every third day Note the pronounced mucification and leucocyte infiltration. Magnification 350 ×

an initial, reversible reaction to a local irritant (5) In the case of MCA induced carcinogenesis at least, invasive lesions may in addition, develop from the basal layer of the epithelium Buds are seen in regions with epithelial atypia as well as in regions with a normal looking epithelium.

The low incidence of epithelial lesions in the control animals included in this study agrees well with the findings in our earlier study (5) There were no obvious differences in incidence between the control groups and the oestradiol or prolactin injected groups, either in short term or long-term experiments. In contrast to this, a combined treatment with both oestradiol and prolactin brought about that the number of animals with buds was higher in the short term experiments. As

it was considered important to know the developmental potentialities of these buds, the long-term experiments were introduced. At 4 weeks after insertion of the MCA thread, the incidence of pronounced cervical squamous carcinomas was high among animals injected with both oestradiol and prolactin, irrespective of a concomitant progesterone treatment. Carcinomas were less numerous and smaller in the control groups, in the oestradiol group, and in the prolactin group. The difference in incidence of carcinomas among animals injected with oestradiol and prolactin (including progesterone) and among those not receiving this combined treatments was statistically highly significant.

Some of the buds in the short term oestradiol prolactin group presented signs of a beginning genuine invasion and, hence, it seems justified to consider the buds as the origin of the overt carcinomas. The size of the 4-week-carcinomas in all the three treatment groups where estradiol-prolactin was used in combination was striking in two cases the carcinomas involved the whole cervical wall to the mesothelial surface. Progesterone could not be demonstrated to be of unequivocal importance for the incidence of carcinomas. The every third-day injection, however resulted in a pronounced mucification in the carcinomas.

Thus, the results obtained in this study demonstrate that treatment of castrated animals with prolactin-oestradiol results in a high incidence of cervical carcinomas. If administered separately the two hormones had no obvious effects and progesterone did not influence the prolactin-oestradiol effect.

In organ cultures of cervical carcinomas from normal animals (olive oil injected animals) oestradiol in different concentrations (1 or 5 µg/ml incubation medium) had no effect on the H-thymidine incorporation (unpublished results) while prolactin produced an effect (6). The latter may be explained by the fact that the culture medium contained foetal bovine serum in which an oestrogen contamination acted synergistically with added prolactin (16). *Heisely* & *Klarins* (25) using a technical procedure similar to ours, did not find any increased incidence of cervical carcinomas in C_3H mice after oestrogen treatment whereas castration was found to reduce the incidence.

The mechanism of synergism between oestradiol and prolactin can only be discussed injected oestrogen may result in an endogenous release of prolactin (12) which together with the exogeneous prolactin may reach a critical serum level oestradiol and prolactin may have affected cell connections and the structure of the basal lamina (13) thus facilitating invasions prolactin may increase the cellular level of the MCA and the oestradiol binding macromolecules (14 24) resulting in an increased uptake of MCA as well as an increased proliferative activity. Provided prolactin in this system influences the intracellular cAMP level this may affect the inducible polycyclic hydrocarbon metabolizing enzymes which may interact with oestradiol induced changes to increase the incidence of carcinomas (10). Finally an oestradiol induced lymphopenia may play a role (7). Before a conclusion has been reached concerning the mechanism of synergism in the cervical carcinomas, comparisons with the oestrogen-prolactin dependent mammary tumours in the rat (13 15) seem to be premature.

A small amount of growth hormone contamination in the prolactin preparation used cannot be excluded. However we have demonstrated that a preparation of anterior hypophyseal hormone(s) acts synergistically with oestradiol in its influence on the incidence of cervical carcinomas. This effect may be of more general nature and may influence the action of different types of carcinogens. Moreover hormones may be a factor of importance in that they may change the pattern of proliferating and non-proliferating cell compartments both in experimental cervical atypias and in cases of human carcinoma *in situ* (22).

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PARTICIPATION OF RENAL, BUT NOT OF SUBMAXILLARY RENIN IN THE HOMEOSTASIS OF THE BLOOD PRESSURE AFTFR EXPERIMENTALLY INDUCED HYPOTENSION IN MICE

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Bing, J. & Poulsen, K. Participation of renal, but not of submaxillary renin in the homeostasis of the blood pressure after experimentally induced hypotension in mice. Acta path. microbiol. scand. Sect. A, 84 391–396 1976.

While hypotension elicited a marked increase in plasma renin concentration in conscious normal mice, no increase was provoked in previously nephrectomized mice in spite of the high renin content of their submaxillary glands. The role of the increased release of renal renin for the homeostasis of the blood pressure was shown by the decrease in pressure which followed blockade of the renin system. Contrary to Saralasin which did not change the blood pressure in nephrectomized mice, injections of SQ 20.881 did in some mice result in a decrease in blood pressure, which was probably caused by its ability to inhibit bradykininases. Both Saralasin and SQ 20 881 elicited marked increases in plasma renin in normal but not in nephrectomized mice, showing that, while renal renin release is controlled by the plasma angiotensin II concentration, this does not apply to submaxillary renin release.

Key words Hypertension, experimentally; renin homeostasis of blood pressure mice.

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Vaso-active substances such as renin, catecholamines and prostaglandins are released in different forms of shock and can thus play a role for the homeostasis of the blood pressure (see *Jakschik et al.* 1974). The role of the renin-angiotensin system as a renal compensation mechanism in hypotension has been postulated by some investigators, but not confirmed by others (see *Powell & DuCharme* 1974). A new way of determining the influence of the renin system is to block its effect by an angiotensin I converting enzyme inhibitor or by a competitive inhibitor of angiotensin II. Whether one or the other form of blockade was used, it was found that the inhibitors block or attenuate the compensatory rise that follows the drop in systemic arterial pressure in dogs with endotoxin and haemorrhagic shock (*Erdös et al.* 1974 *Freeman et al.* 1975). While it applies to most mammals that most of the total body renin is found in the kidneys, the renin content of submaxillary glands is about 60-fold

higher than that of the kidneys in albino mice (see *Page & McCubbin* 1968). The aim of the present study was to see whether the above mentioned effect of the renin system on the homeostasis of the blood pressure in hypotensive states also takes place in mice and, if so, to see whether both renal and submaxillary renin participate in the homeostasis. For this purpose the changes with time in blood pressure and in plasma renin concentration were determined both in normal and in previously nephrectomized mice in which hypotension was induced by injection of dihydralazine or by graded haemorrhage. The importance of the renin system for the homeostasis of the blood pressure was further elucidated by determination of the effect of infusion of a competitive angiotensin II inhibitor (Saralasin) or of injection of an inhibitor of the angiotensin I converting enzyme (SQ 20.881).

MATERIAL AND METHODS

Animals: normal male albino *mice* of the Danish State Serum Institute strain, weighing about 30 g. The mice were *conscious*. A catheter had been inserted *into one or both femoral arteries during a* short term ether anaesthesia at the start of the experiment. After the operation the mice were placed in restraining cages. Some of the mice had been binephrectomized about 18 hours before the experiments.

Hypotension was in some mice induced by graded haemorrhage, most often by repeated removal of 200 µl blood, the total volume being about 800 µl (range 600–1000 µl), which is a mean of about 16 ml/kg. In other mice hypotension was induced by a single injection of dihydralazine (Nepresol® Ciba) . . dose of 0.5 mg/kg was preferential, as higher doses caused a steeper fall in blood pressure with less tendency to reach the plateau which is needed in order to see whether blockade of the renin system causes a further fall in blood pressure or not.

Blockade of the renin system was obtained in two ways. Some mice received continuous i.a. infusion of about 1 mg/kg/h of the competitive angiotensin II inhibitor 1-Sar-8-Ala-Ang II designated *Saralasin* (Norwich Pharm. Comp. New York). Other mice received a single injection of about 3 mg/kg of a synthetic nonapeptide, designated *SQ 20.881* (Squibb Corp., Princeton) which inhibits both the angiotensin I converting enzyme and bradykininases.

Plasma renin concentration, g. ex in Goldblatt Units (G.U.) $\times 10^{-3} \times ml^{-1}$ was determined in plasma from about 20 µl blood drawn from the femoral artery using the capture radioimmunoassay for angiotensin I as described by *Poulsen & Jørgensen* (1974). *Blood pressure* was recorded using a Tybjerg Hansen transducer and a Servogor 511 recorder.

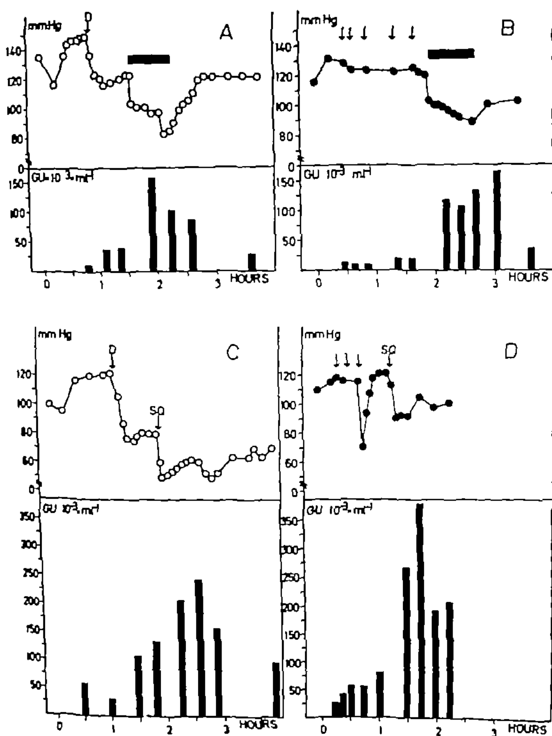
RESULTS

I. Normal mice

The time course of the changes in blood pressure and plasma renin concentration following experimentally induced hypotension in 24 normal mice is shown by representative examples in Fig. 1. It is seen that injection of dihydralazine (O) was followed by a marked fall in blood pressure (Fig. 1A and C). Graded haemorrhage (●) caused a similar fall in blood pressure in some mice, but the fall was only of short duration in other mice (Fig. 1D); in a few mice the blood pressure remained rather uninfluenced by the haemorrhage (Fig. 1B). When the renin system was blocked by Saralasin (A and B) or SQ 20.881 (C and D) there was a further fall in blood pressure followed by a secondary increase, when the blockade was discontinued.

It is further seen that the injection of dihydralazine as well as the graded haemorrhage were followed by significant increases in plasma renin concentration and further marked increases after both types of blockade of the renin system as well as a secondary fall in the values after the discontinuation of the blockade.

Fig. 1. Normal mice. Representative examples of the effect of experimentally induced hypotension followed by blockade of the renin system on blood pressure (mmHg) and plasma renin concentration (Goldblatt Units $\times 10^{-3} \times ml^{-1}$). The hypotension was induced by injection of dihydralazine (O) indicated by an arrow marked D, or by graded haemorrhage (●) indicated by arrows. The blockade was performed by infusion of Saralasin (during the time marked ■ in the two figures at the top) or by injection of SQ 20.881 (at the time indicated by an arrow marked SQ in the two figures at the bottom). The abscissa gives the time in hours.



A
mmHg
D
140
120
100
80
0
GU·10⁻³·ml⁻¹
150
100
50
0
1
2
3
HOURS
B
mm Hg
140
120
100
80
0
GU 10⁻³ ml⁻¹
150
100
50
0
1
2
3
HOURS
C
mm Hg
D
SQ
120
100
80
60
0
GU 10⁻³·ml⁻¹
350
300
250
200
150
100
50
0
1
2
3
HOURS
D
mm Hg
SQ
120
100
80
60
0
GU 10⁻³·ml⁻¹
350
300
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200
150
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50
0
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HOURS

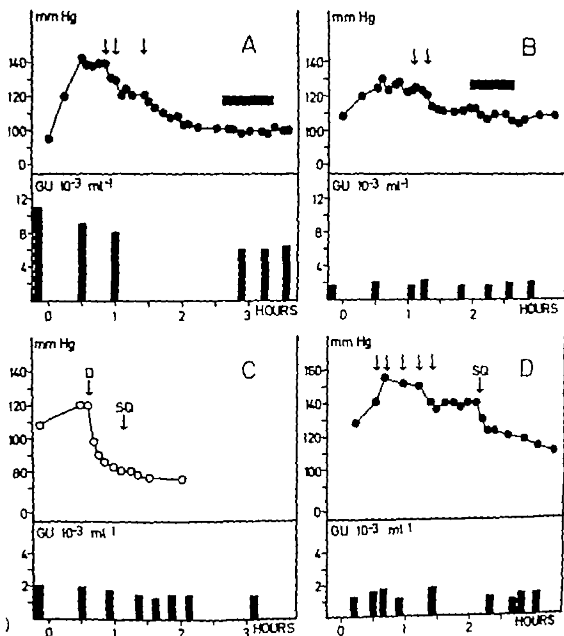


Fig 2 Previously binephrectomized mice The figure gives representative examples of the results obtained in nephrectomized mice. The symbols are the same as those in Fig 1 except the symbols on the scale representing the renin concentration on the ordinates the latter being changed because the renin concentration is lower in nephrectomized than in normal mice.

II Binephrectomized Mice

The results of similar experiments on 16 previously nephrectomized mice are shown by representative examples in Fig 2. The figure shows the experimentally induced hypotension caused a fall in blood pressure similar to that found in normal mice. When graded haemorrhage was followed by infusion of Saralasin there was however no further decrease in blood pressure in the

nephrectomized mice (Fig 2A and B) which is in contrast to findings in normal mice (Fig. 1A and B) If the blockade was performed by injection of SQ 20.881 the result was either that there was no significant effect (Fig. 2C) or a more or less pronounced further decrease in blood pressure occurred (Fig 2D) A decrease in blood pressure after treatment with SQ 20.881 similar to that seen in Fig 2D was observed in two previously nephrectomized mice which had neither been bled nor treated with dihydralazine before the injection of the blocker which was given 2½ hours after discontinuance of the short ether anaesthesia.

In contrast with the marked changes in plasma renin concentration found in normal mice (Fig 1) the renin concentration in the nephrectomized mice would remain uninfluenced by hypotension as well as by blockade of the renin system, as shown in Fig 2A, B, C and D in which the change in ordinate, as compared with that shown in Fig. 1 is caused by the fact that the plasma renin concentration in nephrectomized is lower than that in normal mice.

DISCUSSION

The results presented here show that the renin-angiotensin system plays a role for the homeostasis of the blood pressure in mice, as it previously has been shown to do in other mammals, as mentioned in the introduction. That this participation of the renin system in the homeostasis is due to renal and not to submaxillary renin is shown by the finding that hypotension is followed by a marked increase in plasma renin concentration in normal mice (Fig 1) while this is not the case in previously nephrectomized mice (Fig 2) That the marked increase in plasma renin plays a significant role for the homeostasis is demonstrated by the finding that blockade of the renin system with Saralasin results in a secondary decrease in blood pressure in the normal, but not in the nephrectomized mice When the blockade was performed with SQ 20.881 there was a similar secondary decrease in blood pressure in normal mice. Using this blocker however there was also a secondary decrease in some of the nephrectomized mice, even though the plasma renin concentration remained uninfluenced by the primary hypotension. This difference between the effect of blockade by Saralasin and by SQ 20 881 is probably due to the fact that SQ 20.881 besides being an inhibitor of the angiotensin I converting enzyme, also inhibits bradykininases (for further details see *Davis et al* 1974) and thus augments the concentration of the hypotensive bradykinin in plasma. The present finding that submaxillary renin does not participate in the homeostasis of the blood pressure in mice with experimentally induced acute hypotension does not exclude that submaxillary renin in less acute experiments can play a role for the level of both the plasma renin and the blood pressure, which were found by *Takeda et al.* (1969) to be decreased 40 days after extirpation of the submaxillary glands. Blockade of the renin system by the competitive angiotensin II inhibitor Saralasin results in a rapid, marked increase in plasma renin concentration in normal, but not in nephrectomized mice. This finding is in accordance with our previous (1975) and the present finding, that blockade by SQ 20.881 is followed by an increase in plasma renin in normal, but not in nephrectomized mice. This indicates that, in contrast to renin release from the kidneys, that from the submaxillary glands is not controlled by the plasma angiotensin II concentration.

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POLYVINYLPYRROLIDONE-STORAGE DISEASE

Light Microscopical, Ultrastructural and Chemical Verification

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Reske-Nielsen, E., Bojsen-Møller M., Vetner M. & Hansen, J. C. Polyvinylpyrrolidone-storage disease. Light microscopical, ultrastructural and chemical verification. Acta path. microbiol. scand. Sect. A, 84 397–405 1976.

The light and electron microscopical findings in the polyvinylpyrrolidone-storage disease are reported on the basis of biopsies of skin, striated muscle, bone marrow and liver from one patient and a subcutaneous nodule from another patient. Both patients suffer from diabetes insipidus and have been treated for several years with Insipidin retard® which contains polyvinylpyrrolidone (PVP) as the retarding agent. Deposits of PVP have been demonstrated in all the tissues examined and can easily be recognized by certain staining qualities. The combination of haematoxylin-eosin, elastin (Weigert), alkaline Congo red and Sirius red for amyloid and PTAH is specially to be recommended. The ultrastructural findings consist of intracellular vacuoles containing a granular material and probably representing lysosomes. The final identification of the nature of the deposits has been made by spectrophotometric analysis. The suspicion of a case of PVP-storage disease should result in a skin biopsy which usually is sufficient for the diagnosis.

Key words: Polyvinylpyrrolidone-storage disease

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This work presents the pathological and chemical findings in two patients suffering from a disease, due to the deposition of polyvinylpyrrolidone (PVP).

We first became acquainted with this disease when we found deposits of a foreign material in a subcutaneous nodule from a woman with diabetes insipidus. This substance showed the same staining qualities as previously observed in various tissues from another patient with diabetes insipidus (6). As both patients had received Insipidin retard® as daily subcutaneous injections for many years, it was tempting to suggest that the etiology of their new disease was to be found in this drug (3).

Therefore we decided to investigate the possibility that this adjuvans might be identical with the deposited material, and a review of the literature confirmed that such deposits were not unknown (4–15).

Besides the active hormonal substance Insipidin retard® contains polyvinylpyrrolidone (PVP) which is a polymer of vinylpyrrolidone (M.W. range 10,000–700,000) and is

TABLE 1 *Pattern of the Staining Qualities of Polyvinylpyrrolidone (Skin Striat d Muscle Bone Marrow Liver and Subcutaneous Nodule)*

Stain	Colour	Intensity
*Haematoxylin-eosin	grey-blue	strong
van Gieson-Hansen	yellow	faint, uniform
*Elastin (Weigert)	brown-lilac	striking
Mallory PTAH	ochre	striking
*Sirius red (amyloid)	red	striking
*Alkaline Congo red	brown-red	strong
Methyl violet	lilac	faint
Thioflavine T	no fluoresc.	
Periodic acid-Schiff (PAS)	red-violet	strong, but varying
PAS with Diastase	red-violet	strong, but varying
OTAN	bright blue	strong
Gomori's trichrome	rosy	faint
Weil	light brown	faint
Mucicarmine	brown-red	faint
Sudan B	grey-black	faint
Chlorazol fast pink	pink	uniform

* These five stains are recommendable.

Negative stains Congo red, Da enport, Giemsa, Perls' method for haemosiderin, Reticulin, Scharlach rot, Toluidine blue Unna-Pappenheim, van Kossa.

Examination for immunofluorescence of the skin from patient 1 is negative (anti γ-globulin, antifibrinogen, anticomplement, antialbumin and antilipoprotein (sign. Erik Sommer Hansen, the University Institute of Pathology Aarhus))

used as a vehicle for different drugs to retard their effects (11) PVP is also used as a tablet binding and granulating agent, and it is not metabolised in the organism. It is excreted in urine and faeces but a substantial amount is stored in the reticuloendothelial system following the long term administration (>10 years) of high-molecular PVP (MW >50 000) (15)

MATERIAL AND METHODS

Case 1 A 45-year-old man with diabetes insipidus following traumatic lesion of the base of the brain at the age of 20 years. For 25 years he has been treated daily with 2 ml Insipidin retard® (Vasopressinum NFN Polyvidonum NFN) administered as 2 subcutaneous injections.

When he was 38 years old his left tibia was fractured in an automobile accident, and 2 years later he sustained a spontaneous fracture of the left femoral neck Both fractures showed difficulties in heal g Almost at the same time as the second fract re the patient had observed diminished strength and sen itivity in both hands.

Neurologica amination revealed signs of a polyneuropath I e skin was atrophic and scaly with numerous bluish papules the size of a pinhead. As the pathogenesis of the neurological disease could not be determined, in spite of intense clinical investigation, a skin-muscle-nerve biopsy was performed.

Case 2. A 46-year-old woman with an 11 year history of diabetes insipidus, probably as a result of Boeck s disease (verified by chest X-ray in the same year)

Since January 1963 she had been treated with Insipidin retard® with a daily dose f 0.6 ml subcutaneously During the past year she had developed several subcutaneous nodules outside the injection areas. A nodule from the chest wall was removed for examination.

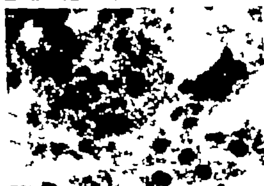
The biopsy material included skin, the left long palmar muscle, bone marrow and l er tissue from the first patient, and the aforementioned subcutaneous nodule from the second patient.

A portion of the biopsies from all the localisations were used for paraffin sections. The staining procedures performed ppear from Table 1 Fat staining was performed on frozen sections of skin and liver tissue from patient 1 and of the subcutaneous nodule from patient 2 Part of the striated muscle was treated according to Koelle's cholinesterase method to visualize the subneural apparatuses and intravital staining with methylene blue to demonstrate the intramuscular nerves (13)









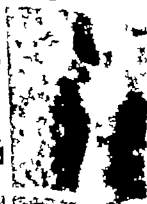










Fig 1 Skin with numerous phagocytes containing conspicuous

Fig. 2. Transverse section of striated muscle. Between the ... vessel walls and the nerve. Haematoxylin-eosin, × 125.

Fig. 3 Bone marrow Note the large number of reticulum ... Sirius red for amyloid, × 300

Fig 4 Liver The Kupffer cells are distended by a brown-lilac

Fig. 5 Subcutaneous nodule with a giant cell and many ... teristic grey-blue colour of PVP Haematoxylin-eosin, × 125

Fig. 6 The same subcutaneous nodule in chlorazol fast pink,

For electron microscopy of striated muscle, liver and the subcutaneous nodule, the specimens were fixed in glutaraldehyde 2 per cent and osmium tetroxide 1 per cent dehydrated and embedded in Vestopal W. Five blocks were taken from each specimen, and from each block six grids were examined. The grids were stained with uranyl magnesium acetate and lead citrate and examined in a ZEISS EM 9.

Part of the tissue from patient 2 was analysed for a chemical confirmation of the presence of PVP. A spectrophotometric determination of PVP iodine-complex as described by *Larkin & Kupel* (1965) was used.

RESULTS

The basic lesions in all the tissues examined are identical and consist of strange deposits. The resulting changes, however differ according to the tissue involved.

Light Microscopy

From Table 1 it will appear that the deposits have a characteristic pattern of staining qualities. In haematoxylin-eosin stained specimens large deposits are conspicuous, whereas scattered PVP bearing phagocytes can be overlooked. In Weigert's stain for elastin the deposits stand out clearly from the unstained background. The PTAH gives several shades of ochre to identify the PVP material, and with the Sirius red and the alkaline Congo red stains for amyloid the deposits are obvious.

It is particularly to be noted that the substance is stained by some methods used for demonstration of amyloid (7 14) but the specific fluorescence with thioflavine is not present, and there is no green dichroism with the alkaline Congo red stain. Moreover we would like to call attention to the chlorazol fast pink method which is recommended for polyvinylpyrrolidone by *Pearse* (1972)

The skin: numerous phagocytes filled with granules are to be found around vessels, hair follicles, sweat and sebaceous glands throughout the dermal layer (Fig 1). The tissue response to this material is only slight and consists of a few lymphocytes around some vessels and focal hyperplasia and degeneration of the epidermal layer.

Striated muscle: deposits are seen in phagocytes of the connective tissue between fascicles and muscle fibres. In vessels they are situated in the endothelial cells as well as in fibroblasts of the wall. Finally polyvinylpyrrolidone is noticed in Schwann cells of the intramuscular nerves (Fig 2). There is no sign of inflammation, but fields of small angular fibres are seen, expressing a neurogenic atrophy. This pathological feature is also reflected in the degenerative and regenerative changes of the subneural apparatuses and the intramuscular nerve fibres.

Bone marrow: the paraffin section contains numerous reticulum cells filled with the same material lying among normal cells of the haematopoietic tissue (Fig 3).

Liver: in the needle biopsy large amounts of PVP are present as visualized in the Fig 4. There is no inflammatory response, but a slight degree of steatosis is seen.

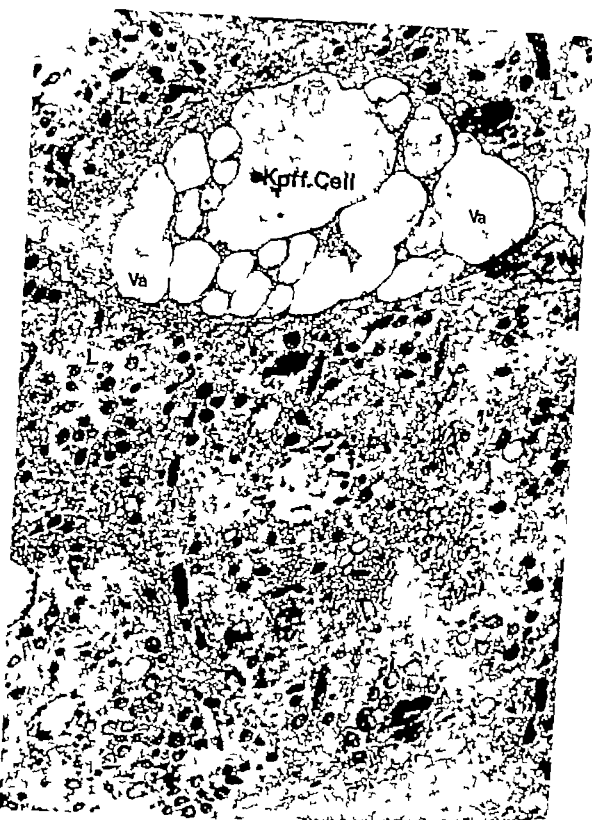
Subcutaneous nodule: macroscopical examination reveals a 4 × 2 × 1 cm very firm piece of tissue with a yellow-spotted cut surface. Microscopical examination shows foreign body granulomas separated by a dense fibrous connective tissue. In each granuloma there are numerous mononuclear and multinuclear cells containing an abnormal substance which shows the characteristic staining pattern of PVP (Fig 5) and also has the special colour with chlorazol fast pink (Table 1 Fig 6).

Fig 7 Longitudinal section of the [illegible] × 6000.

a) A phagocyt with many vacuoles of different sizes and shapes is seen between the upper two fibres. In the vacuoles there are fine granules together with a few coarser particles (arrows). Note that these particles are concentrated along the limiting membranes of the vacuoles. The nucleus of the phagocyte is degenerated.

b) Small vacuoles (arrows) are present in the cytoplasm of endothelial cells. In the upper right corner part of a phagocyte is seen.

MF: Muscle fibre Ph Phagocyte Va Vacuole; Nu Nucleus of the phagocyte V Vessel EC: Endothelial cell


L
Kpff.Cell
Va
Va
L
L

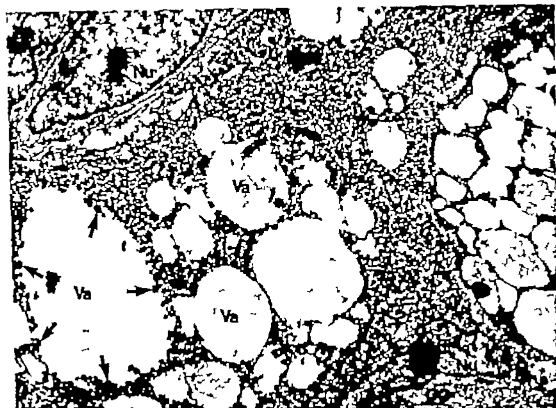


Fig 9 Ultrastructure of the subcutaneous nodule, × 6000 The giant cell contains vacuoles of varying sizes. Along the limiting membranes coarse particles (→) are seen while some of the vacuoles contain a fine granular material.
N Nucleus of the giant cell Va Vacuole; CO: Collagen

Electron Microscopy

Striated muscle vacuoles of different size are a conspicuous feature in the electron micrographs. They are situated in the cytoplasm of endothelial and perithelial cells and in macrophages between muscle fibres. The vacuoles are limited by an osmiophilic distinct membrane and some of them contain very small particles or/and a threadlike substance, whereas others are clear with scattered electron dense granules along their limiting membranes (Fig 7)

Fig 8 Ultrastructure of liver tissue × 6000. The upper part of the picture shows a Kupffer cell distended by vacuoles containing granules and particles as in the muscle tissue.
Kpf. Cell Kupffer Cell; Va: Vacuole L Liver cell K Nucleus of Kupffer cell.

Liver vacuoles identical to those observed in striated muscle tissue are located inside the cytoplasm of the Kupffer cells which may be distended to such a degree as to compress the liver cells (Fig 8)

Subcutaneous nodule in the giant cells several vacuoles are seen, and they may be distended by tiny granules. Along their limiting membranes coarse particles are often seen (Fig 9) The connective tissue separating the granulomas is normal. Fibrils characteristic for amyloid has neither been found in this tissue nor in the striated muscle or liver

Spectrophotometric Analysis

Subcutaneous nodule 639 mg of the tissue (260 mg dry weight) was analysed. The result is 12 mg PVP/g dry weight.

DISCUSSION

The literature concerning polyvinylpyrrolidone gives no evidence for damage from *oral* intake (11 15) and the question about its carcinogenic effect seems no longer debatable as several patients have received injections of PVP-containing drugs for many years without development of neoplasms (15). *Blecher & Burnette* (1968) state that no evidence at all has been found of metabolism nor of deleterious effects from storage. Contrary to this opinion a few other authors describe that the storage of PVP resulting from *injections* may produce severe clinical manifestations (1 4 5 8, 10)

The symptoms and signs can be attributed to deposits in skin, joints, bones, lungs, liver and kidney Our cases confirm these observations with regard to skin, bone and liver and furthermore, patient 1 developed a polyneuropathy while patient 2 had some subcutaneous nodules outside the injection areas. To our knowledge these phenomena have not been reported earlier

The basis for the clinical manifestations in our patients is confirmed by histological examination, which reveals deposits of polyvinylpyrrolidone in all the biopsies. A remarkable finding is the localisation of the substance in the intramuscular nerves, which gives a complete explanation of the polyneuropathy

Table 1 shows the results of the panel of stains used by us. Of these we have selected five stains, namely haematoxylin-eosin elastin (Weigert) PTAH, and the alkaline Congo red and Sirius red stains for amyloid. If only small deposits are present, the Weigert stain is particularly to be recommended.

Some authors (4 5 10 12) recommend the chlorazol fast pink stain as the best and most permanent method of identifying PVP but in our opinion the above mentioned combination of stains gives a special pattern for the substance in question, and enables the deposits to be more distinctly visualized. Finally the stability of these 5 stains can be vouched for as the slides from patient 1 are now 5 years old.

The ultrastructure corresponds to the electron micrographs in the article of *Bert et al.* (1972) The material is situated in vacuoles of different sizes, probably lysosomes. We suggest that the different size and shape of the vacuoles, with their tendency to rupture or melt together may be attributed to the great hygroscopic property of PVP The *localization of the PVP-granules in the vessel* walls is interesting as it suggests a transport mechanism. This transport mechanism gives a key to a treatment proposed by *Bert et al* (1972) In a patient with this storage disease they mobilised PVP from the deposits into the blood using colchicine and cortisone. Afterwards a peritoneal dialysis was carried out resulting in a small decrease of the PVP plasma values. This procedure takes a long time and is stressing to the patient but on account of the high molecular weight of the PVP it is not possible to eliminate it through the kidneys.

The diagnosis of the PVP-storage disease can be made by means of a skin biopsy and by the characteristic colour pattern which appears from Table 1 Ultrastructural examination is not necessary A spectrophotometric determination to confirm the pathological findings is recommended.

We are indebted to the staff at the Department of Neuropathology and dr *Martin R. Lower* Department of Ophthalmology Aarhus University Hospital for excellent assistance.

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IS THERE A DIURNAL VARIATION IN THE SUSCEPTIBILITY OF MOUSE SKIN TO THE TUMORIGENIC ACTION OF METHYLCHOLANTHRENE?

A Study of Tumour Yield with Special Reference to the Variation Between Cages

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Iversen, O H. & Iversen U M Is there a diurnal variation in the susceptibility of mouse skin to the tumourigenic action of methylcholanthrene? A study of tumour yield with special reference to the variation between cages. Acta path. microbiol scand. Sect. A, 84 406–414 1976.

Skin tumour development was studied in groups of mice painted once with 125 μg of 3-methylcholanthrene (MCA) either at 12·00 or at 24 00 MET Eight animals were kept in each box. The animals were observed weekly for 20 months and all tumours were registered. There was no difference between the two groups of mice as regards tumour induction time or number of papilloma-bearing mice In the groups of mice treated at 24·00 the number of skin tumours to develop was 9 per cent higher than in groups of mice treated at 12·00. This difference in papilloma yields is not statistically significant Among female mice painted at 24 00 carcinoma-bearing animals were significantly more numerous (50 per cent) than among those painted at 12·00 whereas there was no difference between the groups of male mice. Considering the groups collectively (males + females) the intergroup difference (17 per cent) in advantage of painting at 24·00 was barely significant ($0.5<p<0.10$) There was no difference between the groups as regards the total number of carcinomas to occur When the tumour yields in individual boxes were recorded, the results were found to vary greatly The slight increase in tumour yield after night painting correlates with the circadian variation in proliferative activity of the epidermis. Previous reports in the literature have shown similar differences. Further investigations and better methods seem necessary before a definite conclusion can be drawn concerning a possible diurnal variation in the susceptibility of mouse skin to chemical carcinogenesis. It is also emphasized that it is necessary to exercise great caution when the results of classical epidermal chemical carcinogenesis experiments are to be interpreted It seems necessary to observe animals for at least 1 months before any conclusion can be drawn

Key words Carcinogenesis, epidermal circadian rhythms hairless mice methylcholanthrene; variation, cages.

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In 1964 *Frei & Ritchie* using the initiation-promotion regimen reported that more skin tumours resulted when the initiating application of DMBA was given at night (22·00) than when it was given during the

day (18:00). A single application of DMBA was followed by promotion by repeated applications of croton oil. The authors suggested that epidermal cells were more sensitive to the carcinogen during DNA synthesis than during other phases of the cell cycle.

Subsequently Iversen et al. reported in 1970 *that the skin tumour yield in hairless* mice was 35 per cent higher after a single application of 125 μg MCA given at 24·00 than after the same treatment at 08·00.

Since the relationship between chemical carcinogenesis and DNA synthesis is of great general interest, it was decided to repeat these latter experiments using larger groups of mice *and different* application times corresponding *more closely to the* highest and lowest rate of DNA synthesis *(Tvermyr 1972)*.

In 1964 and 1965 *Fare* pointed out that the time at which tumours first developed differed significantly in mice kept in different boxes, but given identical carcinogenic treatment. It was presumed that the tumour in*duction* time in a particular mouse was in*fluenced* by the box in which it happened to live.

The aim of this paper *is also to give* a short report on the variations in mice kept in different boxes in epidermal chemical carcinogenesis experiments, and to discuss shortly some possible causes of the variations and the consequences they ought to have on the *planning* of experiments and the interpretation *of results in* epidermal carcinogenesis.

MATERIALS AND METHODS

Animals

Hairless h/hr Oslo mice were used in all the experiments. Spontaneous skin tumours *have not* been observed in these mice. At the beginning of each experiment the animals were 70–90 days old and thus in the resting period *of* hair growth *(Iversen & Iversen* 1967). The animals were housed in plastic boxes, 8 mice to a box, and fed a standard diet and water *ad libitum*. *All the* animals were kept in the same room, with natural light from the windows. The experiments were started in August 1971.

Tumour Induction Experiment

The mice were kept in 24 boxes, 8 animals to a box. Forty-eight males and 48 females were randomly assigned to each of the two experimental groups. 20-Methylcholanthrene (MCA) *was pur*chased from AG Brock (batch no. 8H 119727 40 K). Immediately before use, MCA was dissolved in Benzolum Crystallisabile pro analysi, obtained from E. Merck AG. Using a pipette 125 μg MCA in 0.2 ml of benzene was applied to the dorsal skin, and the animals were held until the benzene had evaporated. One group of animals was treated at 12·00 Middle European Time (MET), the second group at 24·00 MET. After treatment, the animals were examined weekly for 20 months. An out*growth was considered a papilloma* when it measured at least 1 mm in diameter and remained present after two or more examinations. All the animals were kept until death or they were killed because of deterioration *due to* a malignant tumour or other disease. Whenever possible, a necropsy was performed and at least one of the largest tumours from every animal was examined histologically. All lesions *registered as* carcinomas were verified histologically. Infiltration below the musculus panniculus was used as a criterion of malignancy.

RESULTS

Ninety three *mice in each experimental* group survived until the appearance of the first papilloma. The mortality rates trends in the two groups were largely parallel and the numbers of tumours in the two groups could therefore be directly compared, according to standard procedures (tumour yield per num*ber of animals alive at* the time of appearance of the first *papilloma*).

Number of Skin Tumour Bearing Animals

The final number of skin tumour-bearing animals (mice with papilloma[s] and/or carcinoma[s]) is shown in Table 1. The final number of animals with papillomas was about the same in both groups. Among the females, 21 animals in the group painted at midnight developed carcinomas as compared with 14 in the *group painted at noon*. This difference of 50 per cent *is significant* ($p < 0.05$). When males and females were grouped together 34 carcinoma-bearing animals appeared in the group painted at midnight as compared

TABLE 1

Parameters recorded	Time of single application of carcinogen and sex of animals painted					
	Painted at 12·00 MET			Painted at 24·00 MET		
	M	F	Total	M	F	T
Number of animals alive at appearance of first tumour	48	45	93	46	47	
Number of animals with						
Papillomas	44	44	88	43	44	
Carcinomas	15	14	29	13	21	
Mean induction time (until 50 per cent of animals had tumour) in months	6.1	6.6	6·4	6·1	6.2	[illegible]
Total number of tumours:						
Papillomas	247	211	458	263	237	[illegible]
Carcinomas	22	16	38	13	25	
All epithelial tumours	269	227	496	2 6	262	[illegible]
Average number of tumours per animal	5.6	5·0	5.3	6·0	5.6	[illegible]
Mean time of papilloma appearance (latency time) in months	9.2	9.2	9.2	9·1	9.9	[illegible]

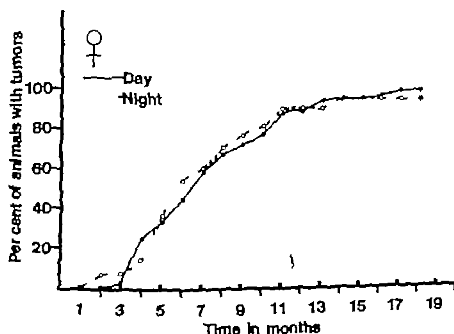


Fig. 1 Percentage of tumour-bearing animals in groups of female mice treated with 125 µg MCA either at 12·00 or at 24·00 MET

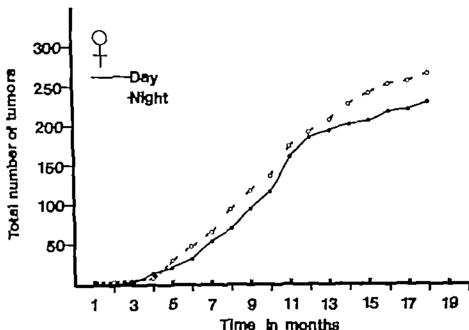


Fig 2 Tumour incidence in groups of female mice treated with 125 µg MCA either at 12 00 or at 24·00 MET

with 29 in the group painted at noon. This difference of 17 per cent is of questionable statistical significance ($p<0.10$) There was no significant difference between the groups as regards the latency time (i.e. until 50 per cent of the animals had tumours) although there was a slight, but statistically non-significant tendency towards a shorter mean latency time in the female mice painted at night.

The percentage of tumour-bearing females as related to time is shown in Fig 1 This figure is also representative of the males. The two curves follow each other closely until the end of the experiment.

Total Number of Tumours

The total final number of tumours are shown in Table 1 Five hundred papillomas developed in 93 mice painted at night as against 458 in 93 mice painted at noon, a difference of 9 per cent. The difference is small, and statistically non-significant when tested by the methods described earlier (*Iversen et al.* 1970) The average number of tumours per animal was consequently slightly but not significantly higher in the group painted at night, and the mean time of papilloma appearance was almost similar in the two groups.

As regards carcinomas, more tumours occurred in the males painted at noon and in the females painted at midnight, but the number was exactly the same in the two groups when the two sexes were grouped together

The total number of tumours as related to time in female mice is shown in Fig. 2. This figure is also representative of the males. The curve for the group painted at midnight runs slightly higher than that for the group painted at noon during almost the whole experimental period.

Variation Between Boxes

Fig 3 shows an example of the percentage of tumour bearing animals in 6 boxes containing female animals, all painted with

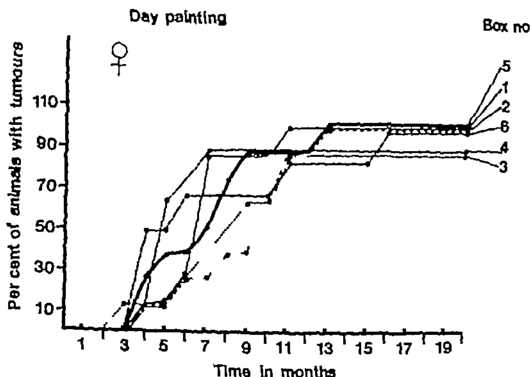


Fig 3 The average percentages per box of tumour-bearing female mice with respect to time after a single application of 125 µg MCA at noon to animals kept under standard conditions with 8 animals in each of 6 boxes.

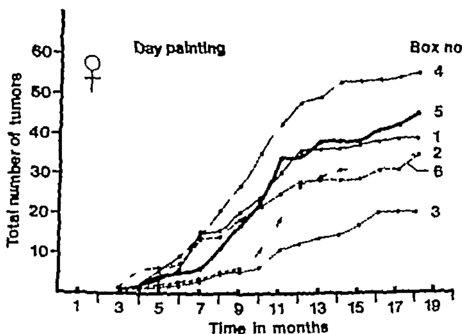


Fig 4 Average tumour incidence per box as related to time after a single application of 125 µg MCA at noon to female anim l kept under standard conditions with 8 animals in each of 6 boxes

MCA at noon. There was only a small variation between the boxes as regards the time at which the first mice became tumour bearing, and there was also a fairly small spread of the results at the end of the observation period. During the period of time between 4 and 12 months, however the rate at which the animals in each particular box became tumour-bearing varied greatly

Fig. 4 shows an example of the total number of tumours in 6 boxes containing female animals, all painted with MCA at noon. There was a considerable variation in the rate at which the tumours appeared in mice in the individual boxes and the spread was most pronounced at the end of the observation period.

Although variations in mice in the individual boxes varied greatly the sigmoid average curves for the whole material are relatively smooth, as shown in Figs. 1 and 2.

DISCUSSION

Halberg (1959) reviewed the phenomenon of circadian rhythms in susceptibility to a large range of external influences. He included the chemical carcinogens among the substances to which there was a varying diurnal sensitivity

In a previous study (*Iversen et al.* 1970) hairless mice were painted with MCA at 24:00 and 08·00 and a significant difference in tumour yield pointed to the advantage of night painting.

The findings in the present study are of doubtful significance, except as regards the number of carcinoma-bearing female animals.

If statistical models are used, two types of errors are possible. One may either erroneously accept differences which are formally significant though they are not real or one may falsely reject real but small differences because they are not statistically significant.

The problem in the present study is whether the theory according to which the sensitivity of epidermal cells to a single application of a chemical carcinogen is greater at night than at day should be rejected because the difference in tumour yield pointing to the advantage of night painting is small and as regards most of the parameters studied, not statistically significant. It is only in the number of carcinoma-bearing females that there is a significant difference pointing to the advantage of night painting

The circadian variations in proliferative activity of hairless mouse epidermis show that cells in mitosis are 2.1/4 times as numerous and that cells in DNA synthesis are 3 times as numerous at 24·00 than at 12·00 (*Tvermyr* 1969 1972) Accordingly if only cells in mitosis or DNA synthesis were sensitive to chemical carcinogens, and if the carcinogen could be applied as a flash dose, the difference between night and day application ought to be large.

If however cells are only slightly more sensitive in mitosis and/or DNA synthesis than in the other phases of the cell cycle, the difference would be reduced.

Furthermore, a polycyclic hydrocarbon carcinogen in benzene dropped on the back skin is not a flash dose. A detailed discussion of the fate of the carcinogens after topical application is given by *Nordén* (1953) and by *Iversen & Evensen* (1962) Many authors have demonstrated how carcinogens remain in the skin for some time after application. An average estimate of the presence of hydrocarbon carcinogens in the epidermis after a single application is 7–9 days (*Engelbreth Holm & Iversen* 1951) *Poel* (1959) studied the fluorescence after a single drop of carcinogen it persisted for 1–3 weeks.

However the so-called ultimate carcinogenic metabolite (*Miller & Miller* 1966 *Dipple et al.* 1968) which is held to react with cellular constituents to start tumorigenesis, may be present in the skin in an effective concentration only for a period of time sufficiently short to make a diurnal study possible. We do not know how many minutes, hours or days after an application "the ultimate carcinogen" is present.

Other biological variations also seem to influence the time of persistence of carcinogens in the epidermis. The "hair cycle

effect" has been extensively discussed by *Engelbreth Holm* and his group (see *Borum* 1954) *Berenblum et al.* (1958) showed that carcinogens remain much longer in the skin if applied during the resting phase than during the growth phase. This may explain the hair cycle effect. *Andreasen & Borum* (1969) have demonstrated that the extent of the early morphological signs of cellular destruction which follow a single application of a moderate dose of DMBA depends on the phase of the hair cycle at the time of application. The primary cell destruction thus depends on the length of time during which the carcinogens or their active metabolites remain in the epidermis.

Another set of factors which may contribute to the masking of genuine differences between the effects of a single application of MCA at diferent times, is the well known fact that, in many respects, the animals in an individual box seem to develop a certain common "biological identity" or similarity in response, while great differences between the boxes always remain.

The present study has demonstrated that the housing conditions may exert a heavy influence on the results of epidermal chemical carcinogenesis experiments if only few animals are used and observation periods are short. Even though each of the boxes contained the same amount of animals, and the animals were kept under standard conditions in a modern animal room, the time at which *50 per cent of the animals became* tumour bearing varied greatly and the same applies to the total number of tumours *Fare* (1965) has discussed in detail the possible causes of such variations. One of these, the different degrees of agressiveness of animals, may be rather important, since it either reflects or leads to differences in stress hormone levels in the blood of the animals, and also to different degrees of scarring and epidermal hyperplasia brought about by fighting

Our findings strongly emphasize that it is necessary to exercise great caution whenever the results of epidermal chemical carcinogenesis experiments are to be interpreted. When the number of tumour bearing animals is counted, it seems necessary to observe the animals for at least 15 months before any conclusions can be drawn. In the period of time between 4 and 11 months, *when so many* investigators finish their experiments, comparison of small groups of mice may lead to deceptive results because of this variation between the boxes.

As regards the assessment of the total number of papillomas the situation is even worse. To obtain a reliable mean value, it seems absolutely necessary to use many animal boxes with a standard number of animals in each of many boxes. Preferably such experiments ought to be repeated at least four times, in order that a mean with a standard deviation of at least four averages of groups of at least 50 animals can be obtained.

Many of the problems in chemical carcinogenesis of mouse skin, for instance, whether the so-called two-stage theory is more correct than the summation theory whether or not the so-called promoters are weak but complete carcinogens whether an initiating but non-carcinogenic dose of a strong complete carcinogen exists whether there is a diurnal variation in the sensitivity of epidermis to a chemical carcinogen, etc., remain under continuous debate because of the conflicting results obtained probably because minor groups of animals commonly are used and they are observed for too short a time.

According to *Tvermyr* (1969 1972) the minimum and maximum of the different parameters of cell kinetics in our strain of hairless mice occur as shown in Table 2. Provided "the ultimate carcinogen" mainly influences the cells shortly after application the present results may be said to give more support to Mottram's original theory of the importance of mitotic activity (*Mottram* 1944 1945) than to the more modern theory of the importance of DNA synthesis for the determination at the cell sensitivity to carcinogens (*Iversen* 1973) *Pound* (1968) points to the importance of the general "state of proliferation" For a more extensive discussion of these problems, see *Iversen* (1973)

TABLE 2. *Circadian Variations in the Proliferative Activity in Hairless Mouse Epidermis*

Kinetic parameters	Time of maximum	Time of minimum
Labelling index	24 00	12-00
Rate of cell entrance into DNA synthesis phase	22-00	14 00
Rate of cell exit from DNA synthesis phase	08 00	16-00
Duration of S phase	12-00	08-00
Mitotic index	24-00	08-00
Mitotic rate	24 00	08:00
Mitotic duration	06 00	02 00

Our previous study of a diurnal variation in the susceptibility of mouse skin to the tumorigenic action of methylcholanthrene due to circadian variation in some of the parameters of cell proliferation, is thus not significantly supported by the present study but it is not refuted either There is a slight tendency showing that the epidermal sensitivity to MCA is higher during the night than during the day The theory is thus still interesting, but for a final conclusion, further investigations and better methods are required.

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THE INFLUENCE OF INFECTION ON THE CONTENT OF LYSOSOMAL ENZYMES IN RAT KUPFFER CELLS AND HEPATOCYTES

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Berg T & Midtvedt, T The influence of infection on the content of lysosomal enzymes in rat Kupffer cells and hepatocytes. Acta path. microbiol. scand. Sect. A, 84 415–420 1976

The activities of β-glucuronidase arylsulphatase A and acid DNAase were measured in homogenates from Kupffer cells and hepatocytes prepared from germ-free monocontaminated and conventional rats. The cells were prepared from a liver cell suspension obtained by treating the perfused liver with collagenase Kupffer cells from germ-free rats were found to have lower lysosomal enzyme activities than cells obtained from conventional rats. Monocontaminated animals (*E. coli* or *Streptococcus pyogenes*) showed intermediate activities. Our data indicate that the level of lysosomal enzymes in macrophages is a function of the endocytic activity of these cells.

Key words Lysosomal enzymes Kupffer cells hepatocytes infection.

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The Kupffer cells of the liver play a central role in the removal of circulating microorganisms (2, 7) Foreign particles that are phagocytosed by Kupffer cells are brought to the lysosomes of these cells and thereby exposed to a variety of acid hydrolases (9) The activities of the lysosomal enzymes in the Kupffer cells would therefore be important factors in the defence against bacteria.

Since the liver macrophages represent only a few per cent of the liver mass (15) it is difficult from studies on whole liver to find out whether changes in the phagocytic uptake of microorganisms are followed by concurrent changes in the activities of the lysosomal enzymes of these cells. Enzymatic methods for the preparation of hepatocytes (6) and Kupffer cells (4) have, however opened up the possibility of measuring the selective contribution of various enzymes from different types of liver cells.

To gain some insight into the mechanism by which Kupffer cells handle bacteria we have in the present investigation compared the activities of some lysosomal enzymes in Kupffer cells derived from germ-free rats and normal, conventional rats as well as from animals contaminated with one species of bacteria (mono-contaminated rats) The activities of the same enzymes have also been measured in purified hepatocytes and in samples from the liver from which the Kupffer cells were derived.

MATERIALS AND METHODS

Animals

Male rats of the CDF strain, aged 100–150 days, weighing 150–225 g, were used.

The germ-free rats were raised and reared as described earlier (12).

Contamination of germ-free rats with either *Escherichia coli* or *Streptococcus pyogenes* was performed as described elsewhere (12 16). The contamination was carried out at least 60 days prior to the experiments. The animals showed a slight diarrhoea the first week after contamination. In all mono-contaminated rats, the numbers of living bacteria present in rectal pellets were determined (13).

The conventional rats were conventionalized for more than 10 generations employing procedures described elsewhere (12). They were kept under standard laboratory conditions and were without any sign of infection.

Chemicals

Pronase (type B) was obtained from Calbiochem, Los Angeles, California. Collagenase (type 1) and enzyme substrates were from Sigma Chemical Co., St. Louis, Mo.

Preparation of Liver Cells

A suspension of liver cells was prepared by treating the perfused liver with 0.05 per cent (w/v) collagenase, as described earlier (5). The liver was first perfused for 3–6 min with Ca^{++} free medium (17). During this period liver lobes were removed for biochemical determinations.

Initial cell suspension (ICS). After the collagenase perfusion the liver was transferred into a Petri dish with suspension buffer (Hanks' solution containing 20 mM HEPES, pH 7.5) and the loosened cells were gently suspended to a final volume of 80 ml.

Parenchymal cells (PC) were purified by low-speed centrifugation of an incubated ICS as described previously (5). The purified cells represented 50–80 per cent of the total number of PC in the ICS.

Nonparenchymal cells (NPC) were obtained by incubating portions of the ICS (2–2.5 × 10^6 PC/ml) at 37 C in the presence of 0.25 per cent (w/v) pronase at pH 7.5 (4). Hepatocytes were selectively destroyed during this treatment and NPC were separated from the debris by centrifugation and repeated washing in Hanks solution containing 20 mM HEPES pH 7.5.

Homogenization of Cells and Tissues

All cell preparations and liver samples were homogenized in cold water in a Dounce homogenizer with 10 down/up strokes of a tight-fitting pestle. Sucrose solutions were added to all homogenates to give a final sucrose concentration of 0.25 M.

Biochemical Determinations

Protein was determined according to the method of Lowry et al. (11). β-glucuronidase (10) was measured at pH 5 with phenolphthalein glucuronic acid (1 mM) as substrate. Acid DNAase (1) was determined at pH 5 with calf thymus DNA (1 mg/ml) as substrate and in presence of 0.15 M KCl. Aryl sulphatase A (1) was measured at pH 4.7 with nitrocatechol sulphate (6 mM) as substrate. 0.1 M acetic acid/sodium acetate served as buffer for all enzymes.

RESULTS

All experiments started within 1 hour after the germ-free or mono-contaminated rats were taken out of the isolators. In the mono-contaminated rats, the numbers of living bacteria present in rectal pellets were found to be at least 10^8 bacteria per gram.

In each experiment cells were prepared from one germ free rat, one mono-contaminated rat and one control animal. The values obtained in the two groups of mono-contaminated rats were of the same order of magnitude. Therefore, these results are grouped together in the following.

The activities of β-glucuronidase, acid DNAase and aryl sulphatase A were measured in homogenates from nonparenchymal cells and hepatocytes as well as from the initial cell suspension and the liver from which the cells were derived.

In Table 1 enzyme activities are expressed as activities per cell (or per g for the whole liver) and in Table 2 as activities per mg protein (specific activity). In Table 1 is included protein content per g liver and per cell for hepatocytes and nonparenchymal cells. The protein content per g liver and per parenchymal cell was almost identical in the three animal groups. Nonparenchymal liver cells from germ free rats had slightly lower protein content per cell than those from mono-contaminated and control animals.

Enzyme activities in liver homogenates. Lysosomal enzyme activities in liver homo-

TABLE 1 *Lysosomal Enzyme Activities and Protein Content in Liver Homogenates and Liver Cells from Germ-Free (GF) Monocontaminated (MC) and Conventional (CONV) Rat*

	GF	MC	CONV	P values lower than a	P values lower than b
a) β-glucuronidase					
Liver	930 ± 40	980 ± 42	965 ± 21	n.s.	n.s.
PC	8.6 ± 0.4	7.5 ± 0.4	8.8 ± 0.1	n.s.	n.s.
NPC	1.2 ± 0.1	1.8 ± 0.3	2.8 ± 0.2	.050	.001
b) Aryl sulphatase					
Liver	1970 ± 80	2470 ± 210	2870 ± 100	10	.001
PC	18.3 ± 1.9	22.5 ± 1.1	19.1 ± 0.7	n.s.	n.s.
NPC	13.6 ± 0.9	16.7 ± 0.3	21.2 ± 1.5	.05	.01
c) Acid DNAase					
Liver	1640 ± 100	1845 ± 181	2110 ± 91	n.s.	.010
PC	10.8 ± 1.4	9.2 ± 1.0	14.2 ± 0.7	n.s.	n.s.
NPC	4.4 ± 0.5	7.1 ± 0.6	9.4 ± 1.0	.01	.01
d) Protein					
Liver (mg/g)	225 ± 9	214 ± 12	208 ± 4	n.s.	n.s.
PC (mg/10^6 cells)	2.03 ± 0.02	1.58 ± 0.13	2.05 ± 0.09	n.s.	n.s.
NPC (µg/10^6 cells)	128 ± 9	144 ± 20	160 ± 9	n.s.	.05

a GF versus MC. b GF versus CONV

Enzyme activities are expressed as n moles/min/g (for the liver) and as n moles/min/10^6 cells (for PC and NPC). Values are means ± s.e.m. for 6 different experiments.

TABLE 2 *Specific Lysosomal Enzyme Activity in Whole Liver Homogenates and Liver Cells from Germ-Free (GF) Monocontaminated (MC) and Conventional (CONV) Rat*

	GF	MC	CONV	P values lower than a	P values lower than b
a) β-glucuronidase					
Liver	4.2 ± 0.2	4.7 ± 0.5	4.6 ± 0.1	n.s.	n.s.
ICS	4.4 ± 0.1	5.0 ± 0.4	4.7 ± 0.1	n.s.	n.s.
PC	4.1 ± 0.2	4.8 ± 0.1	4.3 ± 0.2	n.s.	n.s.
NPC	8.6 ± 0.7	11.2 ± 1.0	17.7 ± 1.6	100	.001
b) Aryl sulphatase A					
Liver	9.1 ± 0.3	12.6 ± 1.0	13.7 ± 0.5	10	.001
ICS	9.4 ± 1.6	13.1 ± 0.5	14.2 ± 1.8	10	10
PC	9.0 ± 1.0	13.1 ± 1.1	11.0 ± 0.9	n.s.	n.s.
NPC	107 ± 16	101 ± 14	137 ± 13	n.s.	.05
c) Acid DNAase					
Liver	7.5 ± 0.6	9.0 ± 0.7	10.6 ± 0.9	n.s.	.010
ICS	7.3 ± 0.5	8.2 ± 0.9	10.9 ± 0.5	n.s.	.010
PC	4.8 ± 0.36	5.4 ± 0.6	6.9 ± 0.3	n.s.	n.s.
NPC	92.5 ± 2.7	47.9 ± 3.6	55.7 ± 4.0	.01	.001

a GF versus MC. b GF versus CONV

Enzyme activities are expressed as n moles/min/mg protein. Values are means ± s.e.m. for 6 different experiments. ICS initial cell suspension, PC parenchymal cells, NPC nonparenchymal cells.

genates, expressed as specific activity or as activity per g of tissue were somewhat lower in germ-free animal than in conventional rats and mono-contaminated animals. This tendency was particularly evident for aryl sulphatase and acid DNAase the specific activities of these enzymes in livers from germ-free rats were about 70 per cent of control values.

Average β-glucuronidase activity (per g or per mg protein) in livers from germ free animals was closely similar to control values, but if hepatic β-glucuronidase activity in each experiment was calculated as per cent of control values, it was found that the specific activity of this enzyme too was significantly reduced ($p<0.05$) in germ free rats as compared with conventional rats.

The specific activity of all enzymes was closely similar in homogenates from liver and from the initial cell suspension in all animal groups (Table 2)

Enzyme activities in hepatocytes and nonparenchymal cells Nonparenchymal cells from germ-free rats were found to have significantly lower enzyme activities (specific activities or activities per cell) than those prepared from conventional animals. The results shown in Table 2 indicate that the activities of all the three enzymes measured were about twice as high in control cells as in cells prepared from germ-free animals. Mono-contaminated animals showed values intermediate between controls and germ free rats.

The activities of two of the three enzymes in hepatocytes were—in contrast to features in nonparenchymal cells—about equal in the three animal groups. Average acid DNAase activity was somewhat lower in parenchymal cells from germ-free rats than in such cells from the other animal groups. The differences were not statistically significant.

DISCUSSION

If reliable information about enzyme distribution in different types of rat liver cells is wanted, it is important that the cells do not leak enzymes during the preparation procedure. Furthermore, the different types of cells should be obtained in relatively high yield otherwise the possibility exists that the final cell preparations are not representative of the total cell populations in the liver It has previously been shown that negligible amounts of lysosomal enzymes as well as cytoplasmic enzymes like LDH, tryptophan pyrrolase or tyrosine aminotransferase are released from the liver during the perfusion with collagenase. Our data show that the specific activities of the three enzymes measured are almost identical in the primary cell suspension and in the liver It is therefore unlikely that selective release of lysosomal enzymes from the cells has occurred during the preparation of the initial cell suspension. Considerable amounts of enzymes are lost into the supernatants during the purification of hepatocytes by low speed centrifugation. However when these supernatants, which are enriched in nonparenchymal cells, were centrifuged so as to sediment all cells, it was found that the cell-free supernatants contained about 10 per cent of the total amount of enzyme in the starting material (the initial cell suspension) This percentage corresponds well with the fraction of cells stainable with Trypan blue It may therefore be concluded that the final purified cells have lost only small amounts of enzymes during the purification procedure. Furthermore, these cells may be incubated in the suspension buffer for several hours without loss of lysosomal enzymes into the medium.

We do not believe that nonparenchymal cells have lost significant amounts of enzymes during the pronase treatment, on the consideration that (1) The cells exclude Trypan blue. This indicates that the membrane is not leaky (3) (2) The enzyme activity per nonparenchymal cell is independent of the time of pronase treatment for at least 4 hours. (3) If nonparenchymal cells are allowed to phagocytose carbon in vivo the carbon remains in the cells after the pronase treatment. Following uptake by phagocytosis carbon probably enters the lysosomes.

At least 50 per cent of both parenchymal cells and nonparenchymal cells in the liver were recovered as purified cells by the methods applied in the present investigation. This high yield of cells makes it likely that the purified cells are representative of the total cell populations in the liver

Our data show clearly that germ-free rats have reduced amounts of lysosomal enzymes in nonparenchymal liver cells. The activities of lysosomal enzymes in hepatocytes seem more unaffected by the germ-free status of the animals. About 10 per cent of hepatic β-glucuronidase and about 20–25 per cent of hepatic acid DNAase and aryl sulphatase are present in the nonparenchymal cells of the liver. In good accordance with this we found that acid DNAase and aryl sulphatase in whole liver homogenates from germ-free animals were reduced relatively more below control values as compared with the activity of β-glucuronidase.

It is reasonable to assume that phagocytes in germ-free animals have a lower endocytic activity than corresponding cells in conventional rats since one of the main phagocytic stimuli in Kupffer cells are bacteria of intestinal origin. It has been shown that Kupffer cells have Fc as well as C_3 receptors (14) These receptors serve as mediators for an effective uptake of foreign particles including bacteria. Germ-free animals have subnormal amounts of γ-globulins probably as a consequence of low antigenic stimulation (18) A result of this would logically be a reduced endocytic activity in Kupffer cells. However even in the gnotobiotic rat the Kupffer cells would not be entirely passive, as it has been shown that senescent erythrocytes (or haemoglobin from such cells) may be digested in liver macrophages. Moreover antigens from the sterilized food may gain access to the circulation. In the mono-contaminated rats, the Kupffer cells may have to take care of bacterial products derived from the monocontaminant. Both *E. coli* and *S pyogenes* are established throughout the intestinal tract (16) *In vitro* it has been shown that mouse peritoneal macrophages respond to increased endocytic uptake of digestible macromolecules by an increased synthesis of lysosomal hydrolases (8)

In conclusion, the low activities of lysosomal enzymes in Kupffer cells of germ-free rats and the somewhat elevated levels in cells from mono-contaminated rats demonstrated in the present report would support the idea that the level of lysosomal enzymes is a function of the endocytic activity of the macrophages.

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DIABETIC CARDIOPATHY

Quantitative Histological Studies of the Heart from Young Juvenile Diabetics

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Ledet, T Diabetic Cardiopathy Quantitative histological studies of the heart from young juvenile diabetics. Acta path. microbiol. scand. Sect. A, 84 421–428, 1976.

A quantitative morphological study at light microscopical level, of hearts from young diabetics and nondiabetics was performed. The groups were comparable with respect to sex-ratio, age, heart weight and blood pressure. Findings in the hearts were as follows: In arterioles in which the luminal diameter ranged between 15–50 μ, a strongly PAS-positive stained vessel wall occurred at a higher frequency among diabetics than among non-diabetics (75 per cent respectively 33 per cent) The PAS-positive structures, however occupied the same area of the vessel wall in the two groups. In arterioles from diabetics, the number of cells in tunica media was increased as compared with that in non-diabetics ($2p<0.01$) The amount of perivascular connective tissue was also increased in the diabetics ($2p<0.01$) There was no indication of an endothelial cell proliferation in the PAS-positive stained vessels from diabetics and non-diabetics.—The wall of the capillaries was not thickened and it was not more PAS-positive in the hearts from diabetics than in those from non-diabetics. Moreover the number of capillaries per square millimeter of heart muscle was the same in the two groups of hearts. In the present light microscopical study the diabetic micro-angiopathy of the heart was demonstrable in the arterioles. The capillaropathy known to occur in other organs was not present in the heart muscle from patients with diabetes of long standing

Key words Diabetic cardiopathy juvenile diabetes histology diabetic angiopathy heart disease.

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The high incidence of heart disease in diabetes mellitus is well-known from epidemiological studies (2, 10, 11) Moreover the myocardial infarction is 2–3 times as common in diabetics as in non-diabetics (14)

In diabetic patients, the heart disease is usually regarded as an expression of atherosclerosis, the development of which is thought to be enhanced by diabetes mellitus" Some factors, however do not fit into this concept

Among non-diabetics, the heart disease is mainly seen in males whereas it occurs with equal frequency in diabetic men and women (2, 3 10 11 14) just as diabetic retinopathy and diabetic glomerulopathy (10) Statistically the incidence of clinical heart disease is related to that of retinopathy (10) and to histologically defined diabetic glomerulosclerosis (4 15) Moreover the degree of PAS-positive changes in the small branches of the coronary arteries is related to the presence of nodular glomruloscleroxis in the kidney (5)

Physiological studies have revealed that the well-known correlation between diabetic micro-angiopathy (retinopathy glomerulopathy) and the duration of diabetes obtains also in cardiac disease (7)

These results suggests that the heart disease in diabetic patients is not only due to atherosclerosis, but also to the specific diabetic angiopathy micro-angiopathy as well as macro-angiopathy

Exact information about the histology of diabetic heart disease is surprisingly scanty and any information about the blood vessels of the heart in young, long term diabetics is not available.

The present report gives one account of quantitative histological studies of the hearts from patients in whom classical juvenile diabetes had persisted for many years. The most striking results obtained include the increase in number of myomedial cells and in the amount of perivascular connective tissue of *small arterioles* There is no *capillaropathy* in the heart muscle of juvenile, long term diabetics.

MATERIAL

Hearts were obtained at autopsy from twelve young subjects all of whom had suffered from classical juvenile diabetes mellitus requiring insulin treatment.

The series comprised five females and seven males (Table 1) with an average age of 38 years and an average duration of diabetes of 23 years. The mean heart weight was 395 gm. and the mean blood pressure was 153/96 mmHg. In five of the diabetics the diastolic pressure was 100 mmHg or more. Diabetic retinopathy had been detected clinically in eleven of the twelve patients and nodular glomerulosclerosis also in eleven of these. Half of the diabetics died from myocardial infarction (Table 1) The resting electrocardiogram was normal in the remaining subjects.

The diabetic hearts were compared with hearts obtained at autopsy from nine non-diabetic subjects (Table 2) The non-diabetic hearts matched with the hearts of diabetics with respect to sex ratio, age and heart weight. One of the non-diabetics had mild hypertension. In four subjects, the blood pressure was not recorded. Five of the non-diabetics died from cardiovascular disease, two from sepsis, and two from poisoning

MORPHOMETRIC METHODS

After fixation in buffered formalin, the wall of the left ventricle was separated from the rest of the heart. A systematic random sampling procedure was used for morphometry The surface of the ventricle was divided systematically into fields of 10 × 10 mm and the tissue blocs from every fourth field were utilized. Tissue blocs in a number of 15–25 were obtained from the left ventricle wall of each heart. The blocs were trimmed into small

TABLE 1 *Juvenile Diabetic Subjects*

	Age years	Sex	Duration of diabetes years	Blood pressure mmHg	Weight of heart g	ECG	Proteinuria g/l	Serum creatinine mg/100 ml
	26	F	16	150/105	260	normal	1	1.2
	27	F	23	150/100	280	normal	+	1.3
	30	F	11	130/90	290	normal	+	1.0
	31	F	29	200/130	390	occl.	2	2.7
	48	F	12	140/80	500	occl.		1.2
	24	M	12	150/100	330	normal	8	1.8
	41	M	28	160/90	540	occl.	5–6	4.3
	44	M	41	140/80	480	occl.	3	1.1
	45	M	29	150/95	420	occl	2.5	1.8
	46	M	24	190/100	340	normal	2–3	3.8
	48	M	16	140/90	410	normal		1.0
	48	M	28	150/90	500	occl.		1.2
Mean	38.2		22.4	153/96	395			1.74
S.E.M.:	2.8		2.7	6.3/3.8	27.7			0.3

TABLE 2. *Non-diabetic Subjects*

	Age years	Sex	Blood pressure mmHg	Weight of heart g	Proteinuria	Serum creatinine mg/100 ml
	26	F	140/90	320	–	0.7
	27	F	140/70	250	–	0.7
	31	F		330		
	32	F		300		
	45	M		340	–	1.2
	48	M	130/85	420		1.0
	48	M		450		
	49	M	160/100	400		1.6
	49	M	120/85	320	–	0.8
Mean:	39.2		142/86	357		1.0
S.E.M.:	3.3		6.6/4.9	23.3		0.1

cubes and then handled by conventional histological technique. Systematical orientation of the tissue blocs during the embedding procedure made it possible to obtain sections where directions of the cut differed as related to the surface of the myocardium. Two sections cut at 7 μm were made from each bloc one was stained with van Gieson-Sirius Red and used for the measurements of connective tissue. The other section together with a section from the kidney was stained with PAS and used for the study of the wall of the small coronary vessels and the glomerulus.

Connective tissue Based on morphological criteria, three types of connective tissue were distinguished in the histological sections:

1) scar-like lesions large irregular areas of connective tissue in the myocardium (Fig. 1) 2) perivascular connective tissue the connective tissue between tunica media and the muscle tissue of the heart (Fig. 2) 3) interstitial connective tissue elongated strands of connective tissue between the muscle fibres without direct connection with scar lesions (Fig. 3)

The connective tissue—defined as the red structures in the van Gieson-Sirius Red stained sections—was estimated quantitatively by point count technique. The histological picture was projected onto a grid with a point density of 63 points per unit area. On each section, the enumeration of points was performed on ten areas selected by systematical random sampling procedure. The number of points on the connective tissue and on the muscle was counted, the pericardium and the endocardium being omitted from the determinations. The total number of points enumerated on each left ventricle ranged from 9450 to 15750 covering from 216 mm² to 360 [illegible] heart tissue. The percentage of connective tissue per unit area heart-muscle was calculated.

Vessels The arterioles were defined as vessels with a tunica media containing at least two cell layers and a luminal diameter 15 μ or more.

The area of the PAS-positive tunica media was also determined by way of a point count procedure in three groups of vessels with a luminal diameter of 1) 15–20, μ 2) 20–30 μ and 3) 30–50 μ. Seven to ten vessels cut at a right angle were estimated in each group of vessels from each person. The measurements were carried out at a magnification of 10 × 100 (oil immersion) with a grid in the ocular. The PAS-positive structures includes inter cellular substances and cytoplasm. The mean area of the PAS-positive tunica media was calculated in square microns per vessel. Also the number of nuclei in tunica media was counted in the vessels utilized for the determination of the area of the PAS-positive structures.

The capillaries, defined as PAS-positive rings with a luminal diameter less than 10 μ, were counted per mm² myocardium by means of the same grid and the same magnification as that used for the study of the arterioles. The enumerations were only performed on fields without scar-like lesions where the capillaries were cut at a right angle. A total of 24 fields from at least 2 tissue sections were [illegible].

The intensity of the PAS-positive staining in the intramural vessels with a luminal diameter between 15–50 μ was assessed semiquantitatively and blindly. The intensity was graduated into + and + +. Also the intensity of the PAS-positive staining and the wall thickness were semiquantitatively evaluated in the capillaries.

STATISTICAL METHODS

The statistical comparison of groups was performed by means of Wilcoxon's non-parametric

test. A 2p-value less than 0.05 was considered as indication of statistical significance.

RESULTS

Connective Tissue

Scar-like lesions were not present in any of the non-diabetics. Such lesions occurred in six of the twelve diabetics. Clinical heart disease had been manifest in these six patients and diabetes had persisted for more than 25 years. The age of the patients with and without scars was not statistically different.

The amount of *perivascular connective tissue* was the same in diabetic hearts with and without infarction (15.4 per cent against 15.9 per cent). There was no relationship between the amount of perivascular connective tissue and heart weight, age and duration of diabetes.

However the diabetic hearts contain a significantly larger amount of perivascular connective tissue than the non-diabetics (Fig 4) ($2p<0.01$)

A certain amount of *interstitial connective tissue occurs* as extremely fine bundles of collagen between the myocardial cells of the normal heart. Morphometrical determinations of this collagen however is impossible at the light microscopical level. Consequently the result includes only clearly visible strains on which the point count technique can be applied. In five of the diabetics in whom clinical heart disease had been manifest, the interstitial connective tissue occupied 0.6–2.7 per cent (mean 1.4 per cent) of the area of the myocardium. Large amounts of connective tissue appeared in only one of the hearts from the non-diabetics it occupied 1.6 per cent of the area of the myocardium in this case





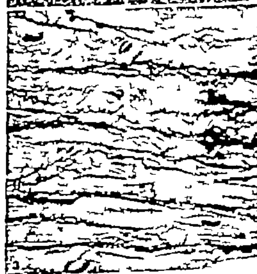


Fig 1 Scar-like lesions. Further explanation, see text.

Fig Perivascular connective tissue. Further explanation, see text.

Fig 3 Interstitial connective tissue. Further explanation, see text.

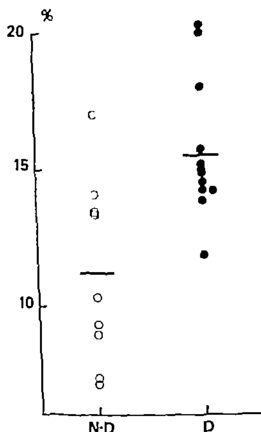


Fig. 4 The percentage amount of perivascular connective tissue in the left ventricle from diabetics and non-diabetics. Each symbol indicates the mean of one heart.

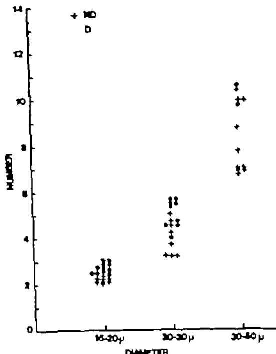


Fig. 5 The number of nuclei in tunica media per cross-section of the arterioles from diabetics and non-diabetics. Each label indicates the mean of one heart.

Intramural Coronary Arteries

The semiquantitative estimate of the intensity of PAS-positive staining gave + + values in 9 of the 12 diabetics, as against in 3 of the 9 non-diabetics. The vessels with the strong PAS-positive intensity were observed in four diabetic subjects without clinical heart disease and in five of these with clinical heart disease. The PAS-positive structures were located in the tunica media.

There was no endothelial cell-proliferation in the small vessels showing accentuation of the PAS-positive reaction.

The area of the PAS-positive tunica media in vessels with a luminal diameter between 15–50 μ was not significantly different in diabetics and non-diabetics (Table 3)

The number of nuclei, however in tunica media of the small intramural vessels was

TABLE 3. *The A ea of PAS-Positi S bstance i the W all of the Small Int amu al Coronary Vessels*

Diameter	Diabetics range	Diabetics mean	Non-diabetics range	Non-diabetics mean
	μ^2		μ^2	
15 20 μ	339–503	(394)	284 414	(350)
20 30 μ	533–940	(715)	536 780	(650)
30–50 μ	1209 2014	(1685)	1013 1967	(1480)

TABLE 4 *The Number of Capillaries per mm² Myocardium*

	Left ventricle		Right ventricle	
	Range	Mean	Range	Mean
Diabetics	2062-2904	(2394)	1725-3042	(2436)
Non-diabetics	2013-3158	(2379)	2263-3042	(2646)

significantly higher in the diabetics than in the non-diabetics (Fig 5 $2p<0.01$) The number of nuclei in tunica media of the small vessels (diameter 15-20 μ) was the same in diabetic subjects with and without clinical heart disease (2.7~2 6) The same held true of vessels with a lumen diameter between 20-50 μ (5.2~4.8)

Capillaries

The number of capillaries per mm² myocardium was the same in the diabetics and the non-diabetics (Table 4) The capillaries were not thicker or more strongly PAS-positive in the presence than in the absence of diabetes.

DISCUSSION

One half of the hearts from young diabetics studied were derived from patients who had presented clinical signs and symptoms of cardiac disease. Such signs and symptoms were not observed in any of the non-diabetics It is very difficult, however to collect a series of hearts from young non-diabetics who have suffered from heart disease. Only very few young subjects have coronary artery disease and, if in evidence, it occurs usually in connection with some rare type of hyperlipoproteinaemia. Consequently if a group of hearts from non-diabetics with cardiac disease has to be culled for comparison with hearts from young diabetics with heart disease, the age of subjects has to be disregarded. In the present study we have chosen to compare non diabetics withot coronary artery disease with diabetics of the same age, although half of the latter ha uffered from clinical cardiac disease.

In the present light microscopical study the attention has been focused on the abnormalities in the capillaries, defined as vessels with a luminal diameter less than 10 μ and in arterioles with a luminal diameter between 15-50 μ. It was a striking feature that there was no thickening of the wall of the capillaries. At light microscopy however the thickness of the capillary wall cannot be exactly measured. Consequently a mild or even moderate thickening of the basement membrane may well have been demonstrable if electron microscopy could have been applied. Still, the fact that thickening of the capillary walls of the heart muscle is not demonstrable by light microscopy is in striking contrast to the findings in many of the other organs in the long-term diabetic body studied (5) It should be noted, however that it applies to most of the light microscopic studies of diabetic micro-angiopathy that little effort has been exercised to distinguish changes in the capillaries from those in the arterioles. Precise information about the prevalence of each of these two types of abnormalities in the individual organs is not available.

In the arterioles studied, a strongly PAS-positive stained vessel wall was seen in 75 per cent of the diabetics and in 33 per cent of the non-diabetics. This conform to the findings obtained by *Blumenthal et al* (1) and *Ledet* (8) in studies of old diabetics. However the difference between the diabetics and non-diabetics in the present investigation was not statistically significant presumably due to the small number of subjects examined.

The area of the PAS-positive structures was the same in vessels from diabetics and non-diabetics. This is in accordance with measurements of the arterioles from old diabetics (8)

whereas it is in contrast to findings in the semiquantitative evaluation by *Blumenthal et al.* (1)

The number of cells in tunica media was significantly higher among the diabetics than among the non-diabetics. This fact has not been recognized previously. It seems to fit with the observation by *Ledet et al.* (9) according to whom diabetic serum was found to stimulate the proliferation of the arterial medial cells in tissue cultures.

In the present study as well as in the one previously reported (8) there was no sign of a proliferation of the endothelial cells in the strongly PAS-positive-stained blood vessels. This is in disagreement with findings in the non-quantitative study by *Blumenthal et al.* (1)

The amount of perivascular connective tissue in the arterioles was higher in the diabetic hearts than in the non-diabetic hearts. There was no indication of a relationship between the amount of perivascular connective tissue, enlargement of the hearts, and occurrence of hypertension in the diabetic group

In the present series of young diabetics all of whom had diabetes of long duration, there was no demonstrable correlation between the amount of perivascular connective tissue in hearts and the duration of diabetes. However such a relationship may well be present if larger series and a wider span of the duration of diabetes were studied.

It is acknowledged that the arterial smooth muscle cells are capable of elaborating connective tissue (6, 12). The arterial medial cell therefore has the potentiality for producing the perivascular connective tissue in hearts of diabetics. The occurrence of interstitial connective tissue in the five diabetics could be a consequence of diseases involving the large vessels as all these patients had been suffering from clinical coronary disease. *Rubler et al.* (13) analysed a small series of four hearts from old diabetic patients. In these cases no stenosis or occlusion of the large coronary arteries was noted. This observation is not necessarily in contradistinction to the present finding of a formation of excessive interstitial tissue which may be quite different in old age. In any case, the cells and the factor(s) responsible for the development of excess of interstitial connective tissue are not known.

Scar like lesions were found only in hearts from diabetic patients in whom clinical heart disease had been manifest. This finding suggests that the scar formation is related to changes in the large extramural coronary vessels.

The functional implications of the appearance of an increased amount of connective tissue in the myocardium are not known. It is conceivable, however, that the large amount of connective tissue contributes to the development of the low stroke volume and the abnormal excercise electrocardiogram in long-term diabetes as described by *Karlefors* (7)

The morphological changes in the arterioles described in the present report are apparently not a consequence of the alterations in the large extramural coronary arteries. There was no indication of a relationship between the clinical heart disease and the presence of intensive PAS-positive staining of the wall, the number of cells in tunica media and the amount of perivascular connective tissue.

In the present light microscopical study the diabetic micro-angiopathy of the heart was demonstrable in vessels with a luminal diameter *larger than* 15–20 μ. These vessels are *strongly PAS positive* but the PAS-positive structures occupy the same area as in the non-diabetics. The number of cells in tunica media is *increased* and the amount of perivascular connective tissue is *increased*. The wall of the capillaries was *not* thickened and the number per mm² is unchanged.

The findings reported in the present paper together with the results of earlier studies of the vessels of the heart in old diabetic patients (8) lend further support to the concept of a diabetic cardiopathy. They also emphasize the fact that diabetic angiopathy present particular features in different organs. In contradistinction to the situation in most

tissues, e.g muscle skin kidney retina there is no light microscopically demonstrable capillaropathy in the heart muscle of patients with long term diabetes.

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EXPERIMENTAL PORCINE NEPHROPATHY CHANGES OF RENAL FUNCTION AND STRUCTURE PERORALLY INDUCED BY CRYSTALLINE OCHRATOXIN A

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Krogh, P. Elling, F., Gyrd-Hansen, N. Hald, B., Larsen, A. E., Lillehoj, E. B. Madsen, A., Mortensen, H. P. & Ravnskov U. Experimental porcine nephropathy Changes of renal function and structure perorally induced by crystalline ochratoxin A. Acta path. microbiol. scand. Sect. A, 84 429–434 1976

Nine pigs were fed crystalline ochratoxin A in amounts corresponding to a feed level of 1 mg per kg for 3 months. The only observable lesion developed was a kidney damage, identical to the naturally occurring porcine nephropathy. The changes of renal function was characterized by impairment of proximal tubular function, indicated by a decrease of the ratio Tm_{PAH}/C_{In} of the ability to concentrate urine, and by an increased urinary excretion of glucose. The decrease of the ratio Tm_{PAH}/C_{In} is correlated with time of exposure to ochratoxin A. The changes of renal structure were characterized by degeneration of the proximal tubules, leading to tubular atrophy accompanied by interstitial fibrosis. At the end of the experiment the kidney liver adipose and muscular tissue of the slaughtered pigs contained sizable amounts of ochratoxin A residues. As the pigs would have passed the meat inspection this represents a possible health problem. The changes observed in this study are identical to those observed by feeding to pigs grains naturally contaminated with ochratoxin A.

Key words Ochratoxin A mycotoxic porcine nephropathy experimental.

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The changes of renal function and structure induced by feeding to pigs a batch of cereal feed naturally contaminated with ochratoxin A have previously been reported (6) Fungal species belonging to the genus *Penicillium* make up a considerable part of the mycoflora of stored grains in Denmark (10) The ochratoxin detected in the above mentioned cereal feed was probably produced

by *Penicillium* strains, such as *P. viridicatum*, *P. cyclopium*, *P. frequentans* or others, known to produce the toxin (5). However these species of *Penicillium* are also known to produce nephrotoxic factors different from ochratoxin (1, 2, 3). The possibility therefore exists that nephrotoxic fungal factors besides ochratoxin A were present in the naturally contaminated feed. In order to elucidate this possibility an experiment has been conducted with conditions identical to the initial experiment (6) except that the only nephrotoxic factor considered was crystalline ochratoxin A.

MATERIAL AND METHODS

Experimental Animals

Nine blocks (litters) of pigs, each comprising two SPF-females, all of Danish landrace, were purchased at 8–10 weeks of age and with a weight of approximately 20 kg. Within the blocks the two pigs were distributed into two groups at random: Control group and experimental group. The pigs were kept at the Experimental Station for Pig Nutrition (Roskilde) and the experimental procedure was as previously reported (6, 8).

Feed

During the experimental period from approximately 25 kg to 90 kg bodyweight (b.w.) the pigs were fed twice a day with daily feed allowance as in (6). The composition of the feed was as follows (in percentage): Barley 79.5, soybean meal 18.0, dicalcium phosphate 1.2, calcium carbonate: 0.8, sodium chloride: 0.4, vitamin-trace mineral mixture: 0.1. The concentration of oxalates in the feed was 262 mg per kg. The individual water consumption was measured daily.

Crystalline Ochratoxin A

Ochratoxin was produced by a strain of *Aspergillus ochraceus* (NRRL 3174) according to a procedure previously reported (7). The crystalline preparation contained 90 per cent ochratoxin A and 5 per cent ochratoxin B, as determined by thin layer chromatography.

Ochratoxin Exposure

The crystalline toxin preparation was mixed with lactose and distributed in gelatine capsules (0.5 ml). The control group was given capsules containing only lactose. The experimental group was given capsules with amounts of ochratoxin A graded according to the body weight, so that the exposure corresponded to a feed level of approximately 1 mg per kg which is identical with group two in the previous experiment (6). Each pig was given one capsule perorally every afternoon until a body weight (live weight) of 90 kg was reached.

Sampling and Analyses of Blood and Urine

Blood and urine samples were collected one week before and one week after the start of the experiment and then every fourth week throughout the experimental period, as previously published (6). The following determinations were carried out on the blood samples: Hematocrit, hemoglobin, albumin, creatinine, α-porcine low-molecular-weight (α-PLMW) protein. Total erythrocyte and leukocyte count, differential leukocyte count and lymphocyte count. The urine samples were analysed for creatinine, glucose (semi-quantitatively), α-PLMW protein, albumin, specific gravity, osmolality.

Renal Function

Measurements of renal clearance (inulin, para-aminohippuric acid (PAH)), of the maximal tubular excretion (Tm) of PAH and of glucose excretion were carried out on 6 pigs from each group 5 and 14 weeks after the start of the experiment, according to (6). Determination of specific gravity and osmolality in urine samples were carried out on all pigs 6 and 13 weeks after the start, according to (6).

Pathology

At the end of the experiment after about 3 months 6 animals (3 from the control group, 3 from the experimental group) were terminated as previously described (6). The left kidney of four of the 6 pigs were fixed by intravital perfusion of glutaraldehyde, after unilateral nephrectomy of the right kidney according to (4). Representative sections of most organs from all 6 pigs were fixed in chilled neutral buffered 10 per cent formalin, and processed as previously described (6). Samples of kidney, liver, striated muscle and subperitoneal adipose tissue were collected for ochratoxin residue analysis. The remaining 12 pigs continue the experiment as part of a long-term toxicological study of ochratoxin A.

Methods of Analyses

Urine albumin was determined by electroimmunoassay according to (9), using an antiporcine albumin antiserum raised in rabbits. All other analyses were performed as described previously (6).

Statistical calculations were carried out as previously described (6) and $P<0.05$ was considered to indicate a significant difference.

RESULTS

Animal Performance

During the period 25 to 90 kg body weight the pigs exposed to ochratoxin were significantly inferior to the control pigs with respect to growth rate and feed conversion. The water consumption was equal for the two groups during the first six weeks, but it was 15-30 per cent higher in the experimental group during the remaining test period.

Blood

The creatinine concentration was significantly increased in the experimental group. During the trial only insignificant differences between the two groups were observed in erythrocyte, leukocyte and lymphocyte counts, hemoglobin and hematocrit.

Renal Function

Small amounts of glucose were found in the urine from pigs in the experimental group after 5 weeks. The average renal clearances of inulin and PAH measured after 5 and 14 weeks did not show any significant difference between the two groups.

Tm_{PAH} and the ratio Tm_{PAH}/C_{I} were markedly decreased in the experimental group after 5 and 14 weeks (Table 1). Glucose reabsorption was also inhibited in this group as indicated by the urine concentration of glucose and especially by the excretion percentage for glucose (Table 1). The ability of the pigs to produce concentrated urine after 24 hours of water deprivation was reduced in the experimental group after 13 weeks but not after 6 weeks (Table 2).

No increase of albumin and a PLMW protein excretion in the urine was observed.

Renal Structure

The kidneys of the three pigs from the experimental group appeared pale and slightly enlarged. Microscopically the lesions in the cortex were focally distributed and consisted principally of degeneration primarily of proximal tubules, tubular atrophy and interstitial fibrosis. In the cortex the majority of the glomeruli appeared normal, but in areas with marked peritubular and periglomerular fibrosis a few hyalinized glomeruli were encountered (Fig. 1). Corresponding to the interstitial fibrosis different degrees of tubular damage ranging from slight dilatation of proximal tubules and reduction in height of brush border to desquamation of tubular cells and marked tubular atrophy were observed (Fig. 2, 3). PAS stained sections revealed thickening of basement membranes of proximal tubules in severely involved areas. The medulla appeared normal. No vascular changes were observed. In the interstitial fibrous tissue eosinophils were frequently noted,

TABLE 1. *Average Maximal Tubular Excretion (Tm) of P-Aminohippuric Acid and Excretion Percentage for Glucose in Six Pigs from Each Group*

Period of exposure (weeks)	Group	Plasma concentration of PAH (µg/ml)	Tm of PAH (mg/min/10 kg b.w.)	$\frac{Tm_{PAH}}{C_{Inul}}$	Plasma concentration of glucose (µg/ml)	Urine concentration of glucose prior to clearance experiment (µg/ml)	Excretion percentage for glucose (%)
5	Control	1208 ± 84‡	20.8 ± 2.7	0.94 ± 0.06	990 ± 60	49 ± 21	0.24 ± 0
	Experimental	1350 ± 122	14.8 ± 4.0*	0.67 ± 0.22*	1077 ± 215	1755 ± 1288*	7.1 ± 6
14	Control	1238 ± 137	20.5 ± 1.8	1.11 ± 0.08	978 ± 124	78 ± 42	0.29 ± 0
	Experimental	1232 ± 398	8.6 ± 4.6	0.51 ± 0.27	988 ± 117	1822 ± 1492	5.0 ± 2

‡ S.D.

* Significantly different from control



Fig 1 Kidney from pig exposed to 1 mg ochratoxin A per kg feed during 3 months, showing a hyalinized glomerulus. PAS-hematoxylin × 600. The kidneys (Fig 1–4) were fixed by vascular perfusion using 1 per cent glutaraldehyde and 2.5 per cent polyvinylpyrrolidone in Tyrode Solution.

Fig 2 Kidney from the same animal. Note the disturbed arrangement of the brush border and the focal interstitial fibrosis which atrophied tubules are seen. PAS-hematoxylin × 250

Fig 3 Kidney from the same animal. Note the desquamated cells (arrow) and the atrophied tubules. PAS-hematoxylin × 350

Fig 4 Kidney from control pig. Note the open tubules and the well arranged brush borders of the proximal tubules. PAS-hematoxylin × 600

TABLE 2. *Average Urine Specific Gravity and Osmolality in Pigs Deprived of Water for 24 Hours*

Period of exposure (weeks)	Group	Specific gravity (× 1000)	Osmolality (mosm/l)
6	Control	1026 ± 2‡	887 ± 64
	Experimental	1026 ± 1	891 ± 34
13	Control	1029 ± 1	992 ± 53
	Experimental	1027 ± 2*	884 ± 85*

‡ SD
Significantly different from control.

but inflammatory changes were never encountered.

No significant lesions were found in other organs and tissues examined.

Residues of Ochratoxin

No ochratoxin was found in any tissue of the three pigs from the control group. Ochratoxin A was found in all the tissues investigated from the three pigs from the experimental group. Increasing concentrations were observed from fat, muscle, liver to kidney (Fig 5). Ochratoxin B and ochratoxin α, a metabolised product of ochratoxin A, were not detected in any tissue.

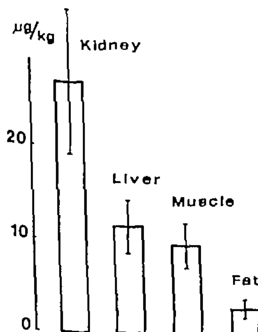


Fig 5 Residues of ochratoxin A in four tissues at 90 kg b w. Each bar represents the mean ± SEM.

DISCUSSION

The results from the present study using crystalline ochratoxin A were close to our recent experiment (6) in which groups of pigs were fed with barley naturally contaminated with varying amounts of ochratoxin A.

In both experiments the pigs demonstrated functional as well as morphological evidence of proximal tubular damage without signs of disease in any other organ or tissue. Furthermore, the character and degree of structural tubular damage, the reduction of urine concentration capacity and the reduction of proximal tubular transport capacity of PAH in the present study was quantitatively similar to the changes seen in the pigs fed with the same amount of native ochratoxin A in the previous study (Table 3). The distribution and concentration of ochratoxin A re-

TABLE 3 *Comparison of Some Effects of Native and Crystalline Ochratoxin A*

Study	I	II
	(In percentage)	
Animal performance		
reduction in daily gain	4.2	5.5
increase of feed units per kg gain	4 9	3.9
Renal function		
reduction of Tm_{PAH}/C_{In} after ca. 3 months	37.9	54 1
reduction of urine osmolality after ca. 3 months, as measured by deprivation of water for 24 hours	12.2	10.9

Study I = the experiment using cereal feed contaminated with 1 mg/kg (native) ochratoxin A (6)
Study II = the present experiment using crystalline ochratoxin A in amounts corresponding to a feed level of 1 mg/kg.
For each study the mean value of the experimental and the control group are compared, and the difference is expressed in percentage.

sidues in the pigs was also in the same range in these two groups.

Thus mycotoxic porcine nephropathy has been completely reproduced by the use of crystalline ochratoxin A, indicating that this substance was the causal determinant in the previous study (6). Ochratoxin A has been found nephrotoxic to all laboratory animals studied (5) and should therefore be considered potentially toxic to man also. The considerable amounts of ochratoxin A found in the carcasses of animals which certainly should have passed the meat inspection may therefore point to a possible public health problem.

Thanks are due to the *State Serum Laboratory* for determination of blood cells, hemoglobin and hematocrit. To Mr *K. A. Christensen*, Royal Veterinary and Agricultural University, for assistance with the statistical calculations, and to the *Department of Veterinary Pharmacy*, *Royal Veterinary and Agricultural University*, for valuable technical assistance.

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Acta path. microbiol. scand. Sect. A, 84 435–439 1976

ROUTINE PROCEDURE FOR DETERMINING GOBLET CELL DENSITY IN THE MUCOSA OF THE RESPIRATORY TRACT

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Mogensen, Chr & Tos, M. Routine procedure for determining goblet cell density in the Mucosa of the Respiratory tract. Acta path. microbiol. scand. Sect. A, 84 435–439 1976

In two normal nasal septa, fine-dissected and stained by PAS-alcian blue whole-mount methods, counts of goblet cells were done in eight different localities in 1 2, 5 10 20, 30 50 and 100 fields measuring 0.01768 mm² The median density and range of goblet cells by each mode of counting were compared and the mean deviation determined. Even as little as 2–5 blindly placed counts, which do not take much time, afford in a 4 mm² area a fairly reliable orientation regarding density considerably more reliable than an estimate of the density in sections, so often reported in the literature. Determination of the goblet cell density can be included as a routine method in the histopathological diagnosis of mucosal diseases of the upper and lower respiratory tract.

Key words Goblet cell density respiratory tract mucosa.

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In studies of individual histological variations or histopathological changes of the mucous membrane of the respiratory tract, it may be desirable to include objective quantitative criteria. A quantitative determination of goblet cell density which was introduced by Moe (1955) for the cat intestine and has later been used by *Kessing* (1968) on human conjunctiva, has been employed in our mucosal laboratory on autopsies and biopsies from the human trachea (*Ellefsen & Tos* 1972 a and b) Eustachian tube (*Bak-Pedersen & Tos* 1972) middle ear (*Tos & Bak Pedersen* 1975) and nose (*Mogensen & Tos* 1975) The methods used are whole mount procedures in which the mucosa in thinned by micro-dissection and the goblet cells are counted on the epithelial surface in circular fields measuring 0.01768 mm Owing to the irregular distribution of the goblet cells (Fig. 1) several counts have presumably to be made to arrive at the true density in the area studied, and this takes time. If the method is to be applicable in daily routine, it must be investigated how far the median density varies in an area when the counts are varied in number and it is essential to find the smallest number of counts that afford fairly representative density in the area concerned. This was the object of the present study

MATERIAL AND METHODS

The material comprises two normal nasal septa, one from a 60-year-old and the other from a 69-year-old woman. The method has been described

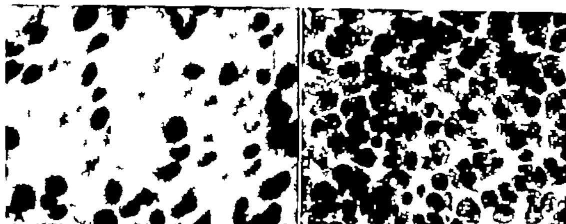


Fig 1 Goblet cells in whole mount. (a) Less density in Case 1 (b) High density posteriorly in Case 2. × 500 (PAS Alcian-blue)

in detail previously (*Mogensen & To 1975*) After PAS-alcian blue whole-mount staining, the entire fine dissected mucosa was embedded in a chamber and the septum divided anteroposteriorly into four quarters. The respiratory region was divided into the lower middle, and upper third. Goblet cells were counted in the four quarters of the middle segment (Fig 2) in 0.01768 mm² circular fields in the Reichardt projection microscope (Visopan) × 500. In each locality the counting fields were placed according to different principles

(1) *Blind placement in the middle of the locality* around a dot of Indian ink (a) one, (b) two, (c) 5, (d) 10 (e) 20 (f) 30 (g) 50 and (h) 100 counts placed according to a given, constant pattern around the dot (Fig 2)

(2) *Blind placement all over the locality* (i) 10 counts, (j) 100 counts scattered all over the locality

(3) *Deliberate placement* of the counting fields One count t the site where the density is estimated to be most representative of the area around the dot of Indian ink (k) and of the total locality (l) Deliberate search for the highest and lowest density in the area around the dot which would be the site of *100* counts (m) and in the *entire* locality (n)

All counts were blind, meaning that the examiner was unaware of the result of previous counts of median density The results were analysed statistically

RESULTS

The median density of all counting experiments in all localities is given in Table 1 the range in Table 2 The median of 100 counts around the dot of Indian ink must be considered the "truest" measure of the density in the central area examined measuring about

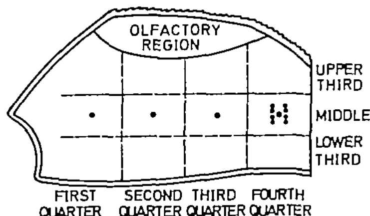


Fig 2 Placement of some counting fields around a dot of Indian ink in the four localities of the septum.

TABLE 1 *Median Density of Goblet Cells in a Differe t Number of Counting Fields*

No. of counting fields	Septum no. 1				Septum no 2			
	First quarter	Second quarter	Third quarter	Fourth quarter	First quarter	Second quarter	Third quarter	Fourth quarter
1 (a)	67	73	59	88	117	116	65	176
2 (b)	59	71	66	80	100	90	60	160
5 (c)	59	69	83	80	93	73	65	165
10 (d)	59	71	82	82	95	75	65	167
20 (e)	55	84	77	84	84	81	71	162
30 (f)	57	76	79	84	87	79	65	166
50 (g)	50	72	75	83	88	79	88	125
100 (h)	49	73	79	83	92	79	88	125
10 (i)	69	70	96	116	81	111	110	177
100 (j)	76	76	85	106	87	96	113	193
1 (k)	62	87	103	117	100	81	74	164
1 (l)	70	69	115	108	74	75	102	135
2 (m)	67	74	87	102	107	93	103	176
2 (n)	70	60	94	122	97	93	106	191

4 mm² To be able to determine divergences from this "true" density when using a smaller number of counts than 100 the median density in each counting group was estimated in relation to the median density found by 100 counts around the dot. These quotients are grouped around the figure 1 The mean deviation in relation to "true" density is very small at 2-50 counts of blind placement (Fig. 3 a) indicating that only a few counts are needed to find the "true" median density within an area of 4 mm² Deliberately placed counts showed some deviation from the "true" density in the area.

When the median of 100 counts scattered blindly over the entire locality (Table 1 (j)) is considered the "truest" density for the *en tire locality* and the deviation from the other counts is calculated as above, it will be seen (Fig. 3 a) that the mean deviation is greatest for 2–100 counts placed centrally in the locality least for deliberately placed counts

TABLE 2 *Rang of Density of Goblets Cells in a Different Number of Counting Fields*

No. of counting fields	Septum no. 1				Septum no. 2			
	First quarter	Second quarter	Third quarter	Fourth quarter	First quarter	Second quarter	Third quarter	Fourth quarter
2 (b)	50–67	69–73	59–83	73–88	89–117	65–116	55–67	144–176
5 (c)	50–68	6 –83	57–88	73–98	79–117	52–99	49–79	106–179
10 (d)	50–68	62–83	57 91	73–101	79–123	52–116	40–84	106–230
20 (e)	33–75	64–103	57–117	72 98	61–110	45–115	41–140	108–197
30 (f)	35–75	62–103	57–117	72–101	61–123	45–116	41–140	106–230
50 (g)	36–62	42–100	30–101	50–101	40–148	50–112	53–183	94–167
100 (h)	32–65	37–100	30–108	50–115	35–145	50–112	53–183	94–172
10 (i)	48–103	28–112	3 –138	76–164	65–142	88–162	68–135	128–208
100 (j)	31–109	20–134	26–184	41–175	33–136	30–178	61–?23	114–302
2 (m)	43–91	38–110	57 118	90–114	62 133	72–114	37–170	150–203
2 (n)	30–110	14–105	28–160	41–204	52–143	33–154	19–193	128–254

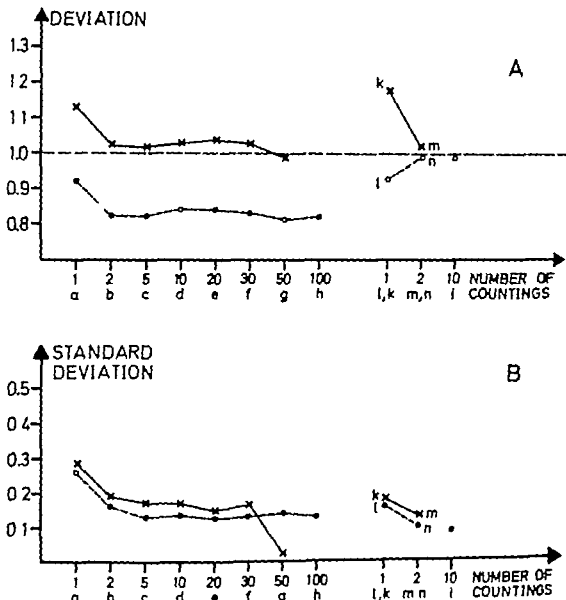


Fig 3 (a) Mean deviation from median density in different modes of counting. Solid line when the median density of 100 counts was estimated as the "true" density of a 4 mm central area. Dotted line when the median density of 100 counts scattered all over the locality is estimated as the "true" density in the locality (b) Standard deviation of deviations in both methods.

(l and n) all over the locality The deviation is very slight for 10 blindly placed counts (l) indicating that the latter gives an equally good impression of the density as 100 counts.

The dispersion on the deviations (Fig 3 b) is the same for most modes of counting if 100 counts are estimate to give the "true" density in the area in the middle of the locality or if 100 scattered counts are estimate to afford the "true" density in the entire locality

DISCUSSION AND CONCLUSION

It has been demonstrated that in a mucosal biopsy whose epithelium rarely measures more than 4 mm² 2–5 blindly placed counts of

goblet cells afford a reasonable certainty that the median density found is close to the "true" density As the preparation of the biopsies is easy and as counting a few fields does not take much time, the method must be considered well-suited for daily routine use. It is appreciably more reliable than an estimate of sections or counting of goblet cells in a high power field where the area studied is extremely small and the cells have been cut.

The area of one locality in the septum averages 170 mm² and the question is then how well the median density of the blindly placed counts within the 4 mm² from the middle of the locality can characterize the density in the entire locality whose "true" density is based upon 100 counts scattered all over the locality Fig. 3 a shows some divergence in all counts, but this divergence is not reduced by increasing the number of counts to 100.

The standard deviation on the divergences (Fig. 3 b) is also not reduced by increasing the number of counts. Thus, the deviation must be considered acceptable, and it may be concluded that the median density of goblet cells on a mucosal biopsy will approximately reflect the median density of the entire locality Examination of 15 noses in smaller magnification revealed only in a few sites limited areas having essentially lower or higher densities than the remaining parts of the locality Accordingly there ought to be little risk of placing the counts in such sites or taking a biopsy from such a site. Septum no. 2 was selected, because its density curve is the most irregular of all the septa studied, in the anteroposterior direction, showing a marked increase in the posterior quarter where the highest density was found, also when compared with all noses studied (*Mogensen & Tos* 1975) In spite of marked deviations, all modes of counting showed considerably higher density in the posterior segment than in the others (Table 1)

No doubt, the examiner's experience and ability to quickly assess whether a blue patch is a goblet cell plays a role in the density determination especially as the goblet cell may show different staining intensity according to its secretory cycle and mucin content (*Moe* 1955) The counts around the dot of Indian ink (a–h) were carried out by one examiner those in the entire locality (i–j) and the deliberately placed counts (k–n) by the other The deviations between the centrally placed and the scattered counts (Fig 3) may be due to the different examiners, although it is not possible to tell, on the basis of the present study who had the more exact results.

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BRIEF REPORTS

METHICILLIN INDUCED NEPHROPATHY

A Case with Linear Deposition of IgG and C3 on the Tubular-Basement-Membrane

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Sommer Hansen, E. & Tauris, P Methicillin-induced nephropathy A case with linear deposition of IgG and C3 on the tubular basement-membrane. Acta path. microbiol. scand. Sect. A, 84 440–442, 1976

A case is reported of methicillin-induced acute interstitial lymphocytic nephritis following treatment of a *St phylococcus au eus* infection. Direct immunofluorescence showed a linear staining of the tubular basement membrane with anti-IgG and -C3 It is suggested that both cellular and humoral immunologic mechanisms are of pathogenic importance.

Key words Methicillin-nephropathy tubular fluorescence; immunologic mechanism.

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In the last few years several cases of acute lymphocytic interstitial nephritis with renal failure have been reported following treatment with methicillin (dimethoxyphenylpenicillin) (1 2, 3 4 5 6, 7 8, 9) On immunofluorescence linear deposition of IgG and C3 on the tubular basement membrane without glomerular deposition has been detected in renal biopsies from some, but by far not all patients with methicillin-associated interstitial nephritis (3 4 5 9)

Within the last 2–3 years we have examined renal biopsies from seven patients with acute renal failure following treatment with methicillin. All biopsies were studied by light microscopy as well as by immunofluorescence. Six of these cases, published elsewhere (9) followed prophylactic methicillin treatment patients undergoing cardiac surgery Th histology wa almost identical in all renal specimens and on immunofluorescence no tubular or glomerular fluorescence was seen. This paper reports ase, which unlike the other six, revealed linear IgC and C3 staining of the tubular basement membrane

Case Report

A 50-year-old man was admitted to the hospital because of an abscess of the right shoulder from which *St phylococcus aureus* was cultured. Methicillin was administrated in doses of 1 g × 6 for 16 days. Following temporary fall in temperature, fever again occurred 3 days after start of therapy and was associated with a morbilliform rash, hematuria and proteinuria. Acute renal failure was seen ten days later serum creatinine increasing from 13 mg/l (at admission) to 60 mg/l and creatinine clearance dropping to a minimum of 27 ml/min. No blood eosinophilia was found. Renal function gradually improved and was normal ten months after discontinuing treatment with methicillin.

Methods

A renal biopsy was obtained 13 days after methicillin treatment was stopped, i.e. 19 days after the onset of the acute renal failure. Part of the tissue was formalin-fixed and paraffin-embedded for histological studies. Immunofluorescent studies were



Fig 1 Linear staining of the tubular basement membrane with antisera against IgG

carried out on cryostat sections of unfixed material from part of the biopsy with antisera for IgG IgM, IgA, IgD and IgE as well as complement (C3) fibrinogen and albumin. Investigation for circulating anti-tubular-basement antibodies in serum from the patient was carried out by incubating normal frozen sectioned kidney tissue with serum, undiluted and in dilution 1 1 1.2, 1 4 1 8, 1 16, 1.32. Details of the technical procedure has been reported elsewhere (10)

Results

By light microscopy severe interstitial infiltration with mononuclear cells was seen Small lymphocytes predominated, but a variable number of plasma cells and eosinophils were also present. In the most severely affected areas there wa marked degeneration or necrosis of the t bular epithel um primarily in the distal tubules. Silver-methenamine-hematoxylin stained sections showed in these areas destruction of the tubular basement membranes The glomeruli were relatively naffected, although some showed slight mesangial widening. Arteries and arterioles were normal.

Direct immunofluorescent st dy revealed linear staining of the tubular basement membranes w th antisera against IgG and C3 (Fig 1) No tubular staining was seen with antisera gainst IgM, IgA, IgD IgE, fibrinogen or albumin N glomerular or vascular fluorescence was observed. Using indirect fluorescence, incubation of frozen sections from a normal human kidney with the patient's serum revealed no fluorescence of tubular or glomerular basement membranes.

Discussion

The clinical picture of methicillin nephropathy with fever rash and eosinophilia as well as the typical time lag between the start of the treatment and the appearance of renal failure has been interpreted as pointing towards an immunologic pathogenesis (3, 9)

The linear staining of the tubular basement membranes with antisera against IgG and C3 in our case corresponds well to the 2 cases reported by Baldwin et al. (1) and Border et al. (3) These authors also demonstrated the presence of a methicillin antigen along the tubular basement membrane (TBM) and circulating anti-TBM-antibodies in the patient's serum detected by an indirect IF-technique. These studies might indicate that methicillin or methicillin-deriv tes were bound to the tubular basement membranes as a hapten and subsequently lead to formation of antibodies re active with TBM. In contrast to the above mentioned studies, w were not able to demonstrate circulating anti-TBM-antibodies against normal (not hapten-conjugated) TBM.

It is still not clear why only a few patients with acute interstitial nephritis show presence of immunoreactants in the TBM. Six of our 7 cases were negative. This also applies to several cases reported in the literature (1 2 3) Technical error does not seem to be responsible since our negative cases were investigated together with other biopsy specimens (e g from patients with glomerulonephritis) which showed positive results using the same batches of antisera. The explanation could either be that binding of immunodeposits is of limited duration or that an antibody reaction against TBM is not operative in all cases of methicillin nephropathy The severe interstitial mononuclear infiltration (which is very similar to acute allograft rejection) might indicate a cellular immunologic mechanism as the main responsive factor

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THE RAPID-FLOW CYTOFLUOROMETRIC IDENTIFICATION OF TUMOR CELLS IN EXFOLIATED CELL POPULATIONS

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The presence of tumor cells in exfoliated cell populations from the cervix of mice could be readily displayed with rapid-flow cytofluorometry

Key words. Exfoliated cells tumor cells rapid flow cytofluorometry

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In a previous work (1) the cytofluorometric characteristics of exfoliated cervico-vaginal cells from normal mice and from 3 4-benzpyrene-treated mice having various degrees of cervical atypias were described The different subpopulations, displayed via a multichannel analyzer were numerically characterized by their mean channel location and coefficient of variation. The present report deals with the identification of tumor cell populations in suspensions containing exfoliated cervico-vaginal cells from mice. The ultimate objective is

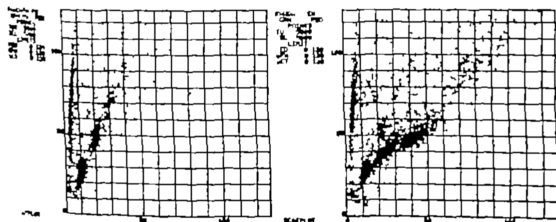


Fig 1 a. Cytofluorogram of exfoliated cervico-vaginal cell populations from 10 normal C57 Bl mice. The horizontal axis depicts acridine orange induced green fluorescence and the vertical axis acridine orange induced red fluorescence. The 3 heavy dotted areas correspond to intermediate-superficial cells (upper left) parabasal cells (upper right) and leukocytes (below)

Fig 1 b The addition of tumor cells to suspensions similar to those of Fig. 1 a resulted in new dotted subgroups in the cytofluorogram (extreme right) The new subgroups represent hyperdiploid and hypertetraploid ascites tumor cells

to find out the minimum concentration of tumor cells in cervico-vaginal cell suspensions, which can be detected in this way

Cervico-vaginal cells from 10 C57 Bl normal mice were pooled in a tube containing 5 ml fixative (absolute methanol 45 ml concentrated acetic acid 10 ml a.d. 100 ml distilled water) The suspensions were stored at 4 C and processed as described elsewhere (1) Ehrlich s ascites tumor cells of the hyperdiploid-hypertetraploid type were recovered from Balb/c female host bearing a 5-day ascites tumor The ascites fluid was dil ted with Tyrode solution to give the appropriate cell con centrat on. Five ml of this suspension was centrifuged and subsequently fixed in 5 ml of the methanol-acetic acid fixative and stored at 4 C. Al quotes were taken for cytofluorometric measurements and for cytological examination by light microscopy in preparations stained with the Papanicolaou stain. The cells to be analyzed in the cytofluorograph were stained in an acridine orange solution, 5 ppm at pH 7 4

The results depicted in Fig 1 a demonstrate that the normal cervico-vaginal pool of exfoliated cells was composed of 3 distinct cell populations in accordance with previous experiences (1) Each one was readily registered in the cytofluorogram one group corresponded to superficial-intermediate cells, other to parabasal cells and the third to leukocytes. The addition of Ehrlich ascites tumour cells to suspensions of normal cervico-vaginal cells was registered in the cytofluorogram as new subgroups (Fig 1 b)

This preliminary investigation indicates that groups of cells having different cytomorphological characteristics may be recorded in cytofluorograms as separate groups.

Some of the questions which remain t be solved are

a) which is the minimal amount of tumour cells that can be detected with the cytofluorographic technique? and

b) can this detection be applied to similar cell systems in human subjects? These questions are presently being explored at this laboratory

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SELECTIVE LOSS OF BLOOD GROUP ANTIGENS DURING WOUND HEALING

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Dabelsteen, E. & Mackenzie, I. Selective loss of blood group antigens during wound healing. Acta path. mikrobiol. scand. Sect. A, 84 445–450 1976.

Frozen sections of healing wounds of the oral mucosa of 5 rhesus monkeys were examined by a double layer immunofluorescent technic for the presence of blood group B-like antigens. Antigen activity was present in normal epithelium and in the epithelial outgrowth into the wounds but was absent from the outgrowth following treatment with lipid solvents. This finding suggests that glycoprotein but not glycolipid associated antigen is lost from the surface of epithelial cells during wound healing, a pattern of selective loss which may reflect a difference between regenerative and neoplastic lesions.

Key words Wound healing blood group antigens.

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The blood group antigens A, B and H which are located on human erythrocytes are also found in association with other cells throughout the body (13 16 25 26 27). These antigens may be found on the cell membranes of epithelial cells of the mucous membranes as well as in association with mucous secretion (11 25 26) The blood group antigens on erythrocytes are carried on both glycolipid and glycoprotein molecules which are bound to the outer surface of the cell membrane (12, 18 19 22) The majority of these erythrocyte antigens are glycolipid associated but studies indicate that, at least in those individuals who secret antigens in the saliva, erythrocyte blood group antigens may be glycoprotein-bound as well (10) Little is known about the chemical nature of epithelial cell-membrane-bound blood group antigens but biochemical studies of the epithelial plasma membranes of intestinal mucosa indicate that blood group antigens A and B may be bound to both glycolipids and glycoproteins and that both these fractions are decreased or lost in carcinomas (14 15 24)

Biochemical studies reporting loss of antigens in neoplasia are supported by immunofluorescent and immunoperoxidase studies of paraffin-embedded biopsy tissue which show a loss of blood group antigens in premalignant and malignant lesions developing from epithelia in which such substances are normally present, e g., in oral mucosa (2, 4 17 20) in cervical epithelium (7 23) in gastrointestinal mucosa (8, 21) and in pancreas (6)

Recent histological studies of wax-embedded tissues have demonstrated an absence of blood group antigens in the epithelium adjacent to healing wounds, a finding which sug-

TABLE 1 *Controls for Establishing Specificity of Immunofluorescence Staining*

	Human blood group test serum	Conjugate	Results
Monkey oral epithelium and			
Human group B oral epithelium	Anti-A	Anti-human IgG/FITC	—
Human group B oral epithelium	Phosphate buffered saline	Anti-human IgG/FITC	—
Human group B oral epithelium	Anti-B absorbed with B-human erythrocytes	Anti-human IgG/FITC	—
Human group B oral epithelium	Anti B	Labelled normal serum	—
Human group B oral epithelium	Anti-B	Anti-human IgG/FITC	+
*Human group A oral epithelium**	Anti-B	Anti-human IgG/FITC	—

* Buccal mucosa for secretory (1)

gests that loss of blood group antigen activity may be related to cell proliferation and movement rather than to malignancy itself (3) However preparation of tissues for wax embedding involves passage of the tissues through intermediary agents, such as xylene or chloroform, which extract lipid components. Such histological studies therefore appear to have demonstrated only those blood group antigens which survive such processing, that is those antigens which are not extractable with lipid solvents and which probably represent the class of glycoprotein-associated antigens. From these studies it is therefore uncertain whether epithelia adjacent to healing wounds also show loss of lipid-bound blood group antigens as has been reported for carcinomas. As a difference in the expression of lipid-bound antigens between reparative and neoplastic changes might be of diagnostic significance, the present study was undertaken to examine the influence of lipid solvents on the reactivity for blood group antigens of the cells in the epithelium of healing wounds.

MATERIALS AND METHODS

Three rhesus monkeys shown to have an antigen on oral epithelial cells which cross-reacted with human blood group B antigen were used as experimental animals.

Biopsies of stand -dized linear gingival wounds, made by clamping t des spaced by a distance of 1 mm in a haemost were taken one day after wounding. The tissue rozen in liquid nitrogen and 6 µm cryostat sections were cut. Representative sections were stained with haematoxylin and eosin to examine the general histological features of the wound and adjacent epithelium. Blood group antigens were semi-quantitated by titration using a double-layer immunofluorescent staining technique (1 3) using sections which were either 1) air dried or 2) treated for 12 hours in either absolute ethanol, ether xylene, chloroform methanol (1.2) or pyridine to remov lipids. The anti-B serum was an anti B blood group test serum purchased from Hoechst, Frankfurt, Germany The conjugate, the anti-A serum and the fluorescence microscope have been described in previous papers (1 3) The control reactions summarized in Table 1 were used to ensure the specificity of the staining reactions.

RESULTS

The haematoxylin and eosin stained sections of the biopsies showed the classical feature of an early healing wound. From the epithelium of normal appearance adjacent to the wound margin a projection or "tongue" of epithelial cells projected down into the wound (Fig. 1) In the air-dried frozen section, blood group antigens were demonstrated in the normal epithelium, adjacent to the wound as well as in the epithelial outgrowths from the wound margin (Fig 2) The positive reaction was seen as bright green staining of all cell membranes in the spinous cell layer It was not possible by the titration to demonstrate any difference in reactivity of blood group antigens in the outgrowth and the adjacent normal epithelium. The reaction in sections treated with ethanol was slightly less than



Fig. 1 Wounded monkey gingiva one day after wounding. H&E staining. (54 ×)

that of air-dried sections. In all sections treated with the other lipid solvents, no antigen activity was demonstrable in the epithelial outgrowth into the wound (Fig 3) Blood group antigens were present in the epithelium adjacent to the wounded area but the antigen reactivity of the normal epithelium was decreased by three titre steps or more after treatment with lipid solvents (Fig. 4)

DISCUSSION

The results of the present study show that the pattern of antigen-loss in lipid-extracted frozen sections of healing wounds corresponds to that observed in previous histological studies of wax-embedded tissue (5) and demonstrate that the expression of blood group antigens on oral epithelial cells taking part in wound healing is markedly decreased. However a retention of antigen activity was found in unextracted frozen sections. This finding indicates that the loss of blood group antigen activity found in wax-embedded tissues is caused principally by a loss of reactivity for antigens which are not extractable with lipid solvents. At present, it appears reasonable to suppose that the antigens not extracted by lipid solvents correspond to the type of protein-bound antigens which have been isolated biochemically from other types of cell (10 14 15 18) The marked decrease in antigen reactivity in the normal epithelium after extraction with lipid solvents also indicates that the greater part of the blood group antigen in gingival epithelium is lipid associated. The inability to demonstrate a decrease in antigen reactivity in the epithelial outgrowth in unextracted sections could be explained by an insufficient sensitivity of the staining technique to detect differences caused by loss of the small proportion of protein-bound antigen normally present. Alternatively exposure of "cryptic" lipid-bound antigen





Figs. 2 and 3 Wounded monkey gingiva one day after wounding. Immunofluorescent staining for blood group B-like antigen. Area indicated by the right frame in Fig. 1 Fig. 2 Untreated section. Note the positive staining reaction on the cell membranes in the epithelial outgrowth. Fig. 3: Section treated in xylol for 12 hours. No B-like antigens are demonstrated but unspecific staining of epithelial cells is seen (256 ×)

sites as a result of changes produced by wounding in the glycoprotein component of the cell membrane could lead to an actual increase in the reactivity of lipid-bound antigens in the altered epithelium (9)

The results of the present work indicate that the use of paraffin-embedded tissue, as reported in previous histological studies, reveals only blood group antigen not extractable with lipid solvents and that a different distribution pattern may be seen if unfixed frozen sections are examined. Biochemical studies indicate that both glycolipid and glycoprotein bound blood group antigens are lost in intestinal tumors (14 15) The finding of a retention of glycolipid-bound antigen in the epithelium of healing wounds therefore appears to be of interest and suggest the need for investigation of the antigen expression in unfixed frozen sections of oral carcinomas. If glycolipid as well as glycoprotein-bound blood group antigens are lost in these tumors it should be possible to distinguish differing types of antigen loss in wound healing and in malignancy

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Fig. 4 Wounded monkey gingiva one day after wounding. Immunofluorescent staining for blood group B-like antigen. Area indicated by the left frame in Fig. 1 Section treated with ether for 12 hours. Note that no B-like antigens are demonstrated in the epithelial outgrowth (a) whereas B-like antigens are present in epithelium adjacent to wounded area (b) (150 ×)

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PENETRATION OF FLUORESCENT HOMOLOGOUS SERUM PROTEINS INTO THE WALL OF THE AORTA IN RATS WITH ACUTE ANGIOTENSIN HYPERTENSION

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Olsen, F Penetration of fluorescent homologous serum proteins into the wall of the aorta in rats with acute angiotensin hypertension. Acta path. microbiol. scand Sect. A, *84* 451–454, 1976.

A few hours of acute angiotensin hypertension in rats increased the permeability of the thoracic part of the aorta for plasma components. The permeability which was demonstrated by means of homologous circulating fluorescent serum proteins, seemed to be diffuse rather than focal. The fluorescent proteins penetrated into the whole thickness of the aortic wall, but were to a great extent deposited in the subendothelial space and the most luminal part of the tunica media. The deposition took place in the greater part of the circumference of the aortic wall. These results are in agreement with the insudative theory of atherogenesis, and the initial stage seems to be "damming back" of some of the serum or plasma proteins for deposition in the subendothelial space and the most luminal part of the tunica media.

Key words Angiotensin hypertension fluorescent serum proteins aorta rats.

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Penetration of plasma components into the walls of arterioles of animals with acute arterial hypertension, lasting for only a few hours, has been described previously (*Giese* 1961 1964 *Olsen* 1968, *Goldby & Beilin* 1972, *Thorball & Olsen* 1974) This penetration seems to be the initial stage in the development of the hypertensive vascular disease (arteriolosclerosis or arteriolonecrosis)

Arterial hypertension is a well-known atherosclerotic risk factor and by the use of tracers for the native serum proteins or the method of immunofluorescence it has been demonstrated that the permeability of the aortic endothelium is increased during acute experimental hypertension (*Hüttner et al.* 1973 *Shimamoto et al* 1975) Such an increased endothelial permeability might be the initial stage in the development of atherosclerosis.

Therefore, it has been examined whether it would be possible to demonstrate an increased permeability of the aortic endothelium for plasma components after a brief period of acute hypertension by means of a direct method in which labelled circulating homologous serum proteins have been used.

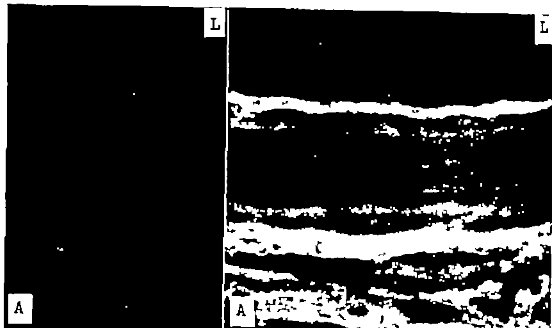


Fig 1 L: lumen, A ad entitia. The aorta from the control rat (left) shows practically no deposition of fluorescent proteins in the wall while the deposition of the fluorescent proteins in the aortic wall from the angiotensin-treated rat (right) is very pronounced and concentrated in the subendothelial space and the most luminal part of the tunica media. Also in the adventitia the fluorescence is strongly apparent. Magnification 600 ×

MATERIAL AND METHODS

Animals White female rats weighing about 150 grams were used.

Anaesthesia A solution of amythal® 25 mg/ml was injected intraperitoneally at the rate of about ¼ ml per 100 grams of rat.

Fluorescent serum proteins were produced as previously described (*Olsen* 1968) and the procedure was a modification of the method of *Nairn* (1962)

Angiotensin Hypertensin CIBA was dissolved in physiological saline.

The experimental te hniq e After induction of complete anaesthesia, a catheter of polyethylene was placed in the femoral vein of each of six rats, and about 40 mg of Lissamine Rhodamine labelled homologous serum proteins was injected intravenously About ten minutes later intravenous injections of angiotensin were started in doses of about ½ microgram each, every fifth minute during four hours. With this procedure, the blood pressure will rise from the normal level (about 90 mm Hg) to 150 160 mm Hg after every injection and normalize before the next angiotensin injection (*Olsen* 1968 After the end of the experimental period he rat were killed with a large dose of amythal int ritoneally and the thoracic part of the aorta wa d in a 4 per cent buffered formalin of pH 7.3

Control animals Four rats otherwise treated in the same way as the experimental rats, were given physiological saline injections intravenously instead of angiotensin, the volumes being identical.

Micr scopical preparations After fixation, the thoracic part of the aorta wa embedded in paraffin, cut into sections five microns thick and examined under a fluorescent microscope

RESULTS

A remarkable difference was observed when the permeation of the fluorescent homologous serum proteins into the aortic wall of the rats treated with intravenous injections of angiotensin was compared with the permeation into the control rats (Fig 1) In the angiotensin-treated animals, the fluorescent proteins had to a high degree penetrated the endothelium and were deposited in the subendothelial space and the most luminal part of the tunica media. The quantity of fluorescent proteins deposited varied from rat to rat, but the depositions in all the angiotensin-treated rats were greatly above those in the control rats. The penetration of the fluores-

cent proteins, when studied in the longitudinal direction of the aorta, seemed to be diffuse rather than focal and occurred in almost the whole circumference of the aorta (Fig. 2)

Besides the very thorough penetration of the fluorescent proteins into the subendothelial space and the most luminal part of the tunica media, there was hardly any doubt that in the angiotensin-treated rats the whole thickness of the wall of the aorta was invaded by the fluorescent proteins to a far greater extent than in the control rats. This observation was possible because the whole thickness of the aortic wall of the angiotensin-treated rats, when viewed under the microscope, showed a very faint specific fluorescence which was non-existent in the control rats. It was observed that when the *total* serum proteins labelled with Lissamine Rhodamine were used, it seemed as if some of the proteins penetrated into the whole thickness of the aortic wall while others were „dammed back" and deposited in the subendothelial space and the most luminal part of the tunica media.

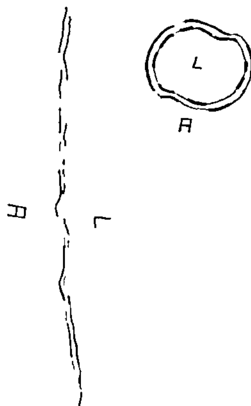


Fig. 2 L: lumen, A: adventitia, thick black lines: Fluorescent proteins. (left) Drawing of a longitudinal section of aorta showing the penetration of the fluorescent proteins which seems to be diffuse rather than focal, and (right) cross section showing the penetration of the fluorescent proteins into almost the whole circumference of the aorta. Magnification 20 ×

DISCUSSION

The results show that a short period of angiotensin hypertension in rats increases the permeability of the thoracic part of the aorta for plasma components. The fluorescent serum proteins were, to a great extent, deposited in the subendothelial space and the most luminal part of the tunica media, and could, owing to a faint specific fluorescence, be recognized in the complete aortic wall. This finding could be interpreted in the way that some of the proteins penetrate easily into the complete aortic wall while others do not, and these are deposited in the subendothelial space and the most luminal part of the tunica media. This difference in regard to penetration into the aortic wall may be explained firstly by differences in the molecular size of the proteins and, secondly by an association between the proteins and the components of the ground substance of the connective tissue in the subendothelial space and the most luminal part of the tunica media (the glycosaminoglycans?)

In normal rats it can be demonstrated that fluorescein labelled bovine serum albumin penetrates the aortic endothelium in small amounts (*Katora & Hollis* 1975) and in acutely hypertensive rats an increased permeability of the aortic endothelium for circulating horseradish peroxidase could be observed while the permeability for ferritin was not so evident (*Hüttner et al.* 1973). Using the method of immunofluorescence, *Shimamoto et al.* 1975 demonstrated an increased permeability of the aortic endothelium in Rhesus monkeys for IgG and beta-lipoprotein when the monkeys were treated

with intravenous injections of different pressor drugs inclusive of angiotensin II for a few hours.

By these different methods it has been possible to demonstrate an increased permeability of the aortic endothelium for plasma components in animals with acute arterial hypertension. Hypertension as a risk factor is thus in agreement with the insudative theory of atherogenesis (for literature see *Walton* 1975)

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THE DIAGNOSIS OF CANCER FROM BODY FLUIDS

A Comparison of Cytology DNA Measurement Tissue Culture Scanning and Transmission Microscopy

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A consecutive series of 70 exudates from 4 patients with clinically suspected malignancy was examined by cytology cytophotometric measurement of DNA, short-term cell culture, scanning and transmission electron microscopy In seven patients (21 fluids) the presence of malignant disease was verified Malignant and benign cases were correctly diagnosed by combination of cytology and DNA analyses. An abnormal DNA profil defined by >10 per cent cells with >2c DNA or single cells with >8c DNA was only seen in malignant exudates. Short-term cell culture with scanning electron microscopy could distinguish between lymphoid cells, histiocytes, fibroblasts, mesothelial cells and cancer cells. Only cancer cells had prominent microvilli on their surface. A future larger series will explore whether a combination of cytology and cytophotometric DNA estimation alone will improve the diagnostic accuracy to the same substantial degree as this pilot study would suggest.

Key words Pleural effusions; cytology DNA measurement; tissue culture scanning microscopy transmission microscopy pleural carcinosis.

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Although cancers diagnosed by serous effusions are seldom curable (13) and the average survival time of patients with tumour cells in body fluids is very short (15) accurate diagnosis of malignancy remains an important goal. Recent studies have dealt with the application of various new methods. DNA measurements have been found of variable value (4 11) Transmission electron microscopy in combination with conventional cytology has been claimed to be of value in selected cases (9 16, 18, 22, 24 27) Scanning electron microscopy has only been reported once and had been used mainly as a descriptive tool (9) Cell culture (5 21) studies failed to demonstrate consistent differences between normal and malignant mesothelial cells. One reason for the limited success of all recent attempts to improve the diagnostic accuracy may be that each single method only has a marginal influence. This pilot study evaluates the respective merits of several techniques applied simultaneously to a

TABLE 1 *Material of Fresh Fluids Studied*

1	M.N 83 years ♀ Abram s bio, mammary adenocarcinoma	2
2	H.N 52 years ♂ Abram s bio lung adenocarcinoma	2
3	L.A.O 44 years ♀ Abram s bio, mammary adenocarcinoma	4
4	O.S 67 years ♂ Abram s bio, lung adenocarcinoma	6
5	G.E. 83 years ♀ autopsy ovarian adenocarcinoma	3
6.	G.L. 32 years ♀ pleura bio, mesothelioma	3
7	H.C. 72 years ♂ Abram s bio, colon adenocarcinoma	1
Total malignant fluids		21
Total benign fluids		49
Total number		70

consecutive series of exudates. The following four special methods were used Cytofluorometric DNA determination short time cell culture, scanning and transmission electron microscopy

MATERIAL AND METHODS

Sixty-five pleural and five fresh ascitic fluids which clinically were suspected of malignancy were studied (Table 1) A further fifteen exudates where the cells were already fixed and stained were also included. All patients were from the Department of Pulmonary Diseases and from the Department of Oncology University Hospital in Uppsala.

Collection of fluids and cell separation. Physicians collected the fluids in sterile heparinized bottles which were left standing for 1 24 hours to allow cells to settle. Most samples were processed within 2 hours. The loose sediment plus adjacent fluid was harvested by pipette and concentrated by centrifugation (1500 × *g* for 10 min) Usually fluids were haemorrhagic and the Ficol density gradient method (10 28) was then used to remove red blood cells.

Preparation of cytologic slides Cell liability was determined by trypan blue. Cells were counted in the Bürker chamber and diluted by phosphate-buffered saline pH 7.2 (PBS) to give 400 000 viabl cells per ml. Six slides were prepared in the cytocentrifuge (1500 × *g* for 5 min) For each slide, 200 µl of suspension was used. Two slides were stained with Giemsa and Papanicolau methods.

DNA measurement Four cytocentrifuged slides were fixed in acetone-ethanol (1 1 for 30 min) The cells were air-dried and stored in a desiccator until staining. Two different methods were used pararosanilin-Feulgen method and ethidium-bromide staining (Table 2) Slides from old exudates were destained according to *Zetterberg & Esposti* (30) upon which the Feulgen method was applied.

Fluorescence measurements were performed by a Zeiss microscope fluorometer using the following filter combination excitation filter BP 546 beam splitter FT 580 and barrier filter LP 590. A uranyl glass standard was used to check the stability of the instrument. Between 25 and 100 cells were measured. Only single and undamaged nuclei were included, otherwise no selection was applied. The data were normalized to 2c (= diploid human cell content of DNA) units and presented in histograms. 2c DNA alues of diploid human peripheral

TABLE 2. *DNA Staining Methods*

Feulgen method	Ethidium-bromid staining
1 Hydrolysis in 4 M HCl at 25 C for 40 min.	1 Incubation in solution of ribonuclease from bo-ine pancreas (50 units) for one hour at 37 C.
2. Wash in 0.1 M HCl.	2. Staining with ethidium-bromide (10 mg/l, pH 7.5 0 1 M in Tris and 0 1 M NaCl at 25 C.
3. Staining with 0.2 per cent pararosaniline solution in 1 per cent potassium metabisulphite and 0.3 M HCl for 2 hrs.	3 Mounting in staining sol tion
4 Wash 5 min in 0 5 per cent Na HSO in 0.05 M HCl for three hrs.	
5 Wash 5 min in 30 per cent, 70 per cent, 96 per cent and 99 per cent thanol	
6. Wash in xylene-eth ol 1 1 and in pure xylen.	
7 Mounting in glycerol	

blood lymphocytes were used as a standard during each measurement.

Tissue culture Specimens were rinsed in PBS after the Ficol procedure and suspended in 5 ml of Eagle's medium with 10 per cent calf serum supplemented with penicillin, streptomycin and fungizone. The suspension was distributed into five Nunc Petri dishes containing sterile cover slips. The dishes were inspected every day rough estimates and descriptions of the morphology under phase contrast were made. Medium was changed twice a week. Living, attached cells were photographed. If cells suspected to be malignant appeared, one dish would be stained with Giemsa.

Transmission and scanning electron microscopy The dishes with living cells were fixed for 24 hours in 2 per cent glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose pH 7.2 and postfixed in OsO_4 90 min. The cells were dehydrated in graded series (50 per cent, 70 per cent) of ethanol, then brought to 70 per cent acetone and further dehydrated to 100 per cent acetone. Cells for scanning microscopy on cover slips were critical point dried from CO_2 and gold coated in vacuum.

Scanning microscopy was usually performed on the same day as the preparation. For transmission microscopy 2 mm² squares of cells were cut out from monolayers using razor blade. The collected cells were embedded in Epon, sections were stained with lead citrate and examined in JEOL 100C electron microscope.

RESULTS

Light Microscopy

The first cytological examination was made without access to a definite diagnosis. This was established later by the comparison of histological examination of biopsies, most often Abram's biopsies, or from the autopsy material (Table 1). All cytological slides were re-analysed in an attempt to find distinctive features which would facilitate identification of the primary origin of the tumour cells.

Mesothelial cells from the patients without malignancy were round or oval, with large often excentrical nuclei with fine chromatin and one or two nucleoli. The abundant cytoplasm was eosinophil or slightly basophil.

Nuclear hyperchromasia and irregular chromatin pattern were important abnormalities seen in all tumours but ovarian adenocarcinoma showed the most prominent changes (Fig. 1). High number of vacuolated cells were seen in our cases of mammary adenocarcinoma and the ovarian adenocarcinoma. The case of ovarian cancer contained, together with isolated vacuolated cells, a high proportion of diplocellular forms (23) which consisted of two tumour cells separated by a large vacuole (Fig. 2).

Compact clusters of cells were seen in mammary cancers. Rosettes and gland-like structures were observed in ovarian cancer. Lung adenocarcinoma did not show any pronounced tendency to form large cell aggregates, but groups of 3–6 cells in rows (Fig. 3).

A useful distinction between benign mesothelial cells and cancer cells could be made by observation of their surface structure. Benign mesothelial cells were smooth whereas cancer cells had an irregular hair-like periphery (Figs. 3, 4).

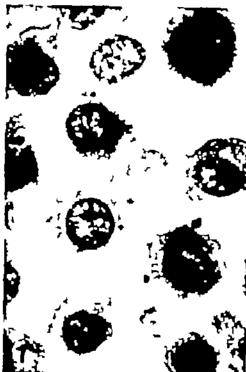
Tissue Cultures

The cell viability of the starting material was high, ranging between 70–90 per cent. Body fluids regularly contained mesothelial cells, histiocytes, lymphoid cells, leucocytes, red blood cells and fibroblasts (Fig. 5), even if they did not contain cancer cells. The different cell types were distinguished by a number of properties (Table 3).

All kinds of malignant tumour cells showed better attachment to glass than to plastic. Mammary and ovarian cancer cells attached within 48 hours; lung adenocarcinoma and colon cancer remained in suspension up to two weeks until some of the cells attached and began to grow. Fibroblasts attached easily, mesothelial cells attached within 48–72 hours. Lymphoid cells and histiocytes had a tendency to remain long in suspension.

The only difference between benign and malignant mesothelial cells was that attachment of the latter was more rapid, otherwise the behaviour and the morphology were similar.

Cancer cells formed groups, often imitating glands. Variability of cell size, shape of the cells and nucleus were seen regularly (Figs. 7, 8); mitoses were observed (Fig. 6) but not frequently.



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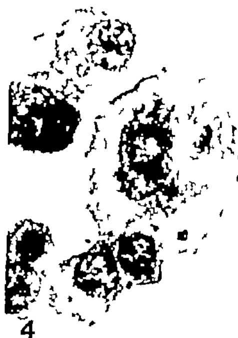
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TABLE 3 *Properties of Exudate Cells in Short Term Cultures*

	Attachment to solid substance	Survival of attached cells	Morphology	Multiplication
Red blood cells	No	–	–	–
Leucocytes	No	–	–	–
Fibroblasts	Very fast, within a few hrs	> 1 month	Spindle	+ +
Mesothelial cell	Within 48–72 hrs	≈ 4 weeks	Spindle, round, horse shoe	+
Histiocyte	Slow up to 1 week	< 1 week	Spherical, hemispherical	+
Lymphoid cells	Slow up to 1 week	< 1 week	Round, clusters	+
Ovarian cancer cells	Fast, within 24–48 hrs	One or two weeks	Cell clusters or epithel-like structures	+ + + +
Mammary cancer cells	Fast, within 24 hrs	Up to 6 weeks	Gland-like structures	+ + +
Lung adenocarcinoma cells	Very slow up to 2 weeks	One week	Gland-like structures	+ +
Colon adenocarcinoma cells	Slow up to 1 week	< 1 week	Gland-like structures	+
Mesothelioma cells	Fast, within 24 hrs	< 1 week	Spindle, round, horse shoe	+

Cancer cells usually died within one or two weeks. All cultures eventually succumbed to infection, apparently because microorganisms had been present already in the original exudate.

Scanning Microscopy

Fibroblasts, lymphoid cells, histiocytes, benign and malignant mesothelial cells and cancer cells could be distinguished. The spindle shaped fibroblasts had an almost smooth cell surface (Fig 11) and a few retraction fibres attached to the bottom of the Petri dish. Lymphoid cells were forming clumps and remained round. The cell surface of lymphoid cells was highly lamellated and villous (Fig 9) Histiocytes were hemispherical and very long and slender cytoplasmatic filaments extended from the attached side of the cell. The free cell surface was highly irregular with numerous lamellae. Characteristically terminal parts of lamellae came to run parallel to the cell surface (Fig. 10) Benign mesothelial cells (Fig 12) were slightly less villous than the malignant ones.

Cancer cells had a striking villous surface, pronounced size and shape variabilities. They had a tendency to form gland-like structures, but isolated cancer cells were also observed. The most irregular cell surface was observed in lung and mammary adenocarcinoma (Figs. 13 14) where the variable lengths of villi were remarkable. Lung adenocarcinoma and

Fig. 1 Nuclear hyperchromasia and irregular chromatin pattern in ovarian carcinoma (case No. 5) Note the irregular cytoplasmic outlines which distinguish these cells from normal mesothelia. Orig. magn. × 100. Immersion oil.

Fig. 2. A diplocellular form from case No. 5 (ovarian carcinoma) Orig. magn. × 100 Immersion oil.

Fig 3 A row of atypical cells from lung adenocarcinoma (case No. 4) Orig magn. × 100. Immersion oil.

Fig. 4 Atypical cells from ovarian carcinoma with clear indications of irregular cytoplasmic outlines. Orig. magn. × 100. Immersion oil.


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mammary cancer cells became flattened. The ovarian cancer cells had a tendency to remain spherical and were covered by numerous, rather regular thick and short microvilli (Fig 15).

Transmission Electron Microscopy

Benign and malignant mesothelial cells had abundant cytoplasm with irregular outlines. Cytoplasmatic processes were short and blunt. The histiocytic cell membrane was irregular with cytoplasmatic ridges of different width and length, running in various directions, very often parallel to the cell surfaces. The plasma membrane of all cancer cells was covered by numerous villi slightly different in different cases. Specimens from both cases of breast cancers were covered by numerous thin microvilli of variable length. The cell membranes of two adenocarcinomas of the lung had prominent microvilli of variable length and width. In our case of ovarian cancer the cells were covered by microvilli of rather short and uniform length, but variable thickness. Cancer nuclei were pleomorphic and often contained irregular nucleoli.

DNA Measurements

Most cells (>90–95 per cent) in pleural and ascitic fluids of non-neoplastic origin are in G1 (11). If, however only mesothelial cells are scored a higher proportion of cells with G2 and S values may occasionally be scored (Fig. 16 panel b). But even in these instances the proportion of mesothelial cells was never sufficiently high to give >10 per cent DNA values outside of 2 ± 0.2c in an unselected cell sample. We therefore regarded all ethidium bromide histograms as abnormal when >10 per cent of unselected cells showed DNA values outside of 2 ± 0.2c.

Different types of abnormalities are illustrated in Fig 16. One type of abnormality is seen in panel c) showing many cells at 4c, but no cells between the 2c and 4c peaks. This probably represents a mixture of stationary normal cells and malignant cells. Panel d) demonstrates how malignant exudates may have an extremely broad spectrum of cells with DNA values ranging from 3–16c.

In all cases of malignant tumour the DNA profile was abnormal. Most of the cancers contained cells with octaploid and higher DNA content, only the ovarian cancer (see case No. 5 Table 1) had a dominance of near tetraploid cells. The case (No. 6) of malignant mesothelioma showed strikingly aneuploid cells. Cells with more than tetraploid content of DNA were never recorded in the benign exudates.

Fifteen additional cases observed in old slides from patients in whom a diagnosis of cancer from body fluids previously had been cytologically established were investigated. In all instances the diagnosis of malignancy could be verified on the basis of abnormal DNA values.

DISCUSSION

The main objective of this pilot study was to test a battery of different methods in a small series of consecutive effusions in order that those which would be most suitable for the daily diagnostic use could be singled out.

The diagnostic accuracy in the present series was good. All malignant cases were correctly diagnosed. Among the benign exudates taken from consecutive series of suspected

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Fig 5. Appearance of cells from benign exudates in culture. The upper arrow points at a mesothelial cell with a well developed ruffling membrane. Lower arrow indicates a histiocyte (compare with Fig. 10). Elongated cells represent fibroblasts and the rounded cells lymphocytes. Orig. magn. × 40.

Fig 6. Living ovarian cancer cells (case No. 5) three days after explantation. Note high mitotic activity in rounded fairly loosely attached cells. Orig. magn. × 40.

Fig 7. Mammary cancer cells (case No. 3) two days after explantation in culture. Epithelial-like island with a few rounded cells which still have not spread on their plastic support. Orig. magn. × 40.

Fig 8. Lung adenocarcinoma cells (case No. 4) three weeks after explantation. Pleomorphic epithelial cells with large nucleoli may be discerned. Orig. magn. × 40.


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tumour cases were a high proportion of samples (60 per cent) where routine cytology gave rise to a suspicion of malignancy (Pap. III). In none of these fluids did subsequent follow-up disclose any malignancy and in retrospect the suspicious cells were evidently mainly "activated" mesothelial cells, i.e. large cells with irregular nuclei and many mitoses.

From patients with verified malignancies, several exudates were obtained where routine cytology was inconclusive. Case No. 5 (Table 1) illustrates this point. The patient's history suggested lung infarction. By way of routine cytology several exudates were evaluated as "slightly suspicious" (Pap III). Cell cultures of the same fluids did, however show attaching, rapidly growing cells of the type seen in Fig. 6. DNA measurements showed a high proportion of 2c (near tetraploid) cells. Cell cultures, DNA measurement, scanning and transmission electron microscopy thus revealed the true nature of the cells. The autopsy disclosed ovarian cancer with only discrete involvement of the peritoneum, but heavy pleural carcinosis.

In the current series each patient did at least on one occasion present an effusion where sufficient DNA abnormalities were picked up in the sample of 25–100 cells. This was, however not true of each single exudate. Examples existed where the proportion of cytologically diagnosed cancer cell was too small to be detected within a random sample of maximally 100 cells.

A combination of factors is probably responsible for the high degree of diagnostic accuracy recorded. The DNA measurements involved a random sample of nuclei. This technique could therefore not pick up a small proportion of tumour cells in the present, relatively small samples of about 100 cells. Cell culture and subsequent examination by scanning and transmission electron microscopy on the other hand, are selective because they are based on the minor fraction of cells which attached to the cover glass. From this point of view the methods complement each other. However cell culture and SEM are time consuming, require constant supervision and are more elaborate. In this series, no additional case was added to the list of malignancies by these procedures. Thus, in the routine, DNA measurement would seem to be sufficient to pick up malignant exudates with substantially better accuracy than cytology alone. It also has the distinct advantage that old slides can be re-examined (30). A series much larger than the present, however is required to explore the full potential and confidence limits of DNA determination of cells in body fluids. It is possible that diploid tumours will be encountered which will register as false negative results (4). However chromosome data indicate that these must be rare. False positive cancer diagnoses cannot be made as long as one of the following two criteria are fulfilled a) >10 per cent of randomly sampled cells with >2c (11) or b) any cells with octaploid and higher DNA values.

The ovarian carcinoma cells were the only ones exclusively represented by near 4c DNA values, whereas all the others including the dertained material had a proportion of cells with octaploid and higher amounts of DNA. The ovarian carcinoma cells were also distinct in scanning electron microscopy.

Cell characteristics and the behaviour of cells in fluids have been described from many aspects, including critical comments and re-evaluation of a huge number of old slides (7 13 14 23). But only little attention has been paid to cell surface description (27). In our study the differences in cell membrane outlines of benign mesothelial cells and adenocarcinoma cells were visible already under

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Fig. 9. Benign lymphoid cells which are rounded and covered by numerous villi. Orig. magn. × 1000

Fig. 10. Typical appearance of a histiocyte with long extended cytoplasmic filaments and a lamellated upper surface. Orig. magn. × 10000.

Fig. 11. Slightly villous spindle-shaped fibroblast with a few ridges parallel to its long axis. Orig. magn. × 1000

Fig. 12. Polygonal benign mesothelial cell with ridges and villi on its dorsal surface.


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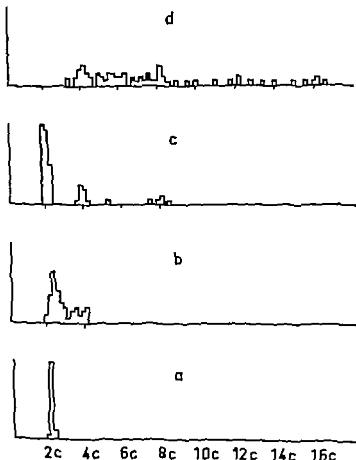


Fig 16 Intercellular distribution of nuclear DNA content (ethidium bromide method) Frequency as a function of DNA content.

a) Human peripheral lymphocytes.

b) Proliferating non-neoplastic mesothelial cells from a case of *probable lung infarction.*

c) Exudate from patient with mammary carcinoma. Case No. 1

d) Exudate from patient with lung adenocarcinoma. Case No. 2.

light microscopy and the difference became still more prominent by scanning and transmission electron microscopy examination.

Body fluids are convenient material for tissue culture, because of the high viability of cells, high number of cells, slight fibroblast contamination which can be still lower if the density gradient separation method is used, and because of possibilities to obtain multiple effusions from the same patients (10) Cells from tissue cultures can be used for further morphological examination of benign and malignant cells (5 10, 21) or they can be used for a number of experiments (6, 10, 29) The main problem appears to be identification of basic cell types, including the origin of cells. Another practical problem which we have met was infection, very often by mycoplasma, although a spectrum of antibiotics was used. All our cultures died within one to eight weeks and we failed to establish permanent cell lines (25)

We have found only one description of scanning microscopy pictures of the cells

Fig 13 Lung adenocarcinoma cells (case No. 4) Densely villous upper surface distinguishes this malignant cell from all types of benign elements encountered in exudates. Orig. magn. × 300 a) × 30000.

Fig 14 Mammary carcinoma cell (case No. 3) displays a villous surface with somewhat shorter villi than that shown in Fig 13 Orig magn. × 6000 a) × 30000.

Fig 15 Ovarian carcinoma cells with villous surfaces. Villi are short and sometimes leaf-like. The cells have retained a rounded shape. Orig. magn. 3000 a) × 10000.

from effusions (9) In that study certain cell types were morphologically different from those described in the present paper This can be explained by the high viability of our cells and the use of improved fixation and preparation methods (3 5) including critical point drying The surface structure of the present lymphoid cells was similar to that of previously described lymphoid cells in culture (25) "Hairy" cytoplasmatic outlines in adenocarcinoma cells could be followed by all morphological methods used. We consider differences in cell surface characteristic of cultivated cells sufficient to distinguish adenocarcinoma cells, lymphoid cells, mesothelial cells and fibroblasts from each other Scanning microscopy is easier and quicker than transmission electron microscopy The specimens could be examined within 48 hours after fixation and a much better survey of the cell surface was also obtained.

In the present series, cytologic examinations were very reliable if complemented with DNA measurements, which was particularly important for the diagnosis of uncertain cases (Pap III) As regards a selected number of cases, tissue culture and scanning microscopy of tumour cells is recommended not only for morphological studies but also as a suitable subject for a number of experiments.

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FIBRINOID NECROSIS OF THE EPITHELIAL CELLS OF THE SKIN

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Sümegi, I Fibrinoid necrosis of the epithelial cells of the skin. Acta path. microbiol. scand. Sect. A, 84 468–476, 1976.

It has been shown in previous studies that fibrinoid necrosis not only occurs in connective tissue and in vessel walls, but can also be observed in the liver cells under special conditions and can also be provoked experimentally It was observed in the present study that certain dermatoses (cases of the herpes group erythema multiforme, drug eruptions, Lyell's toxic epidermal necrolysis, pityriasis lichenoides acuta, and skin affected by UV rays) were associated with "eosinophilic necrosis" in the epithelial cells which morphologically corresponded to the fibrinoid necrosis of the connective tissue and of the liver cells described previously Besides toxic, infectious and septic conditions, circulatory disturbances (hypoxia, anoxia) appear to have a special significance. Observations by way of the light and fluorescence microscope revealed the characteristics of keratin variants and precursors in some necrobiotic cells which influence the peculiar properties of fibrinoid necrosis of the skin epithelia. The role of the mixed paraproteins is emphasized and reference is made to the role of a mixed paraprotein ("keratofibrinoid) which is formed in the course of the regressive process and to which the morphological changes may be attributed.

Key words Necrosis, fibrinoid epithelial cells skin.

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It has been shown in previous studies that fibrinoid necrosis occurs not only in the connective tissue and vascular walls, as has been described so far but it can also be observed in the parenchymatous cells of the liver under special conditions and it may be induced experimentally (25) It has also been found that the various paraproteins that play an important part in such processes are not always present in pure form, but occur mixed. In the study described here, an attempt was made to demonstrate fibrinoid necroses in the epithelial cells of the skin.

MATERIAL

Cases were collected where fibrinoid necrosis was suspected on account of the eosinophilia of necrotic epithelial cells of the skin. One group thus included intra-epidermal vesicles, for instance cases of the herpes group and pemphigus, another comprised cases of erythema multiforme, toxicoderma and drug exanthema. Cases of Lyell's toxic epidermal necrolytic and pityriasis variolиformis lichenoides acuta formed still another group. Punch biopsies of test areas of the skin of patients with actinic sensitivity who were exposed to ultraviolet light were used as "artificial" cases. The control group consisted of patients with pathological keratinization of the epithelial cells (squamous cell carcinoma, trichoepithelioma, molluscum contagiosum, Darier's disease) For a comparison with fibrin and genuine mesenchymal fibrinoid, fibrin-

ous inflammations and vessels from patients with periarteritis nodosa were used.

METHODS

In addition to routine staining the following staining-methods were used: *Imemark's* (12) modification of *Ladewig's* method, *Goldner* (8) *Taft* (modified by *Dahlin* (7)) fibrin staining according to *Weigert Kockel* (14) and by way *Heidenhain's* iron-alum haematoxylin method (11). Other methods were PAS, alcian blue, Congo red, methylgreen-pyronine and thionine, Acridin orange, thioflavin S and rhodamine B according to *Clausen & Dobelstern's* (6) modification were used for fluorochromation and fluorescence microscopy. The sections stained with haematoxylin-eosin and with all the other methods were examined even in Wood's light (19); Congo red stained sections were also examined in polarized light.



Fig. 1. Enlarged, finely acuolar cells in the vicinity of one vesicle of Herpes zoster. Haem.-eosin, × 400.

RESULTS

Since alcian blue never revealed the presence of acid mucopolysaccharides it is not to be further discussed here. Thionine, Congo red, methylgreen-pyronine, thioflavine and PAS gave uncertain results, only increasing the autofluorescence of the formalin fixed sections (19, 25).

Group I

This group included cases of intra-epithelial bullous diseases belonging in the herpes and pemphigus group (herpes zoster, simplex, varicella and pemphigus vulgaris, foliaceus and vegetans).

The herpes and varicella vesicles did not show morphological differences. In all cases, the vesicles were 2–16 days old. The intra-epithelial vesicles were mostly covered with necrotic epithelium, mostly without staining of the nuclei or with shadow cells and with bright red cytoplasm in preparations stained with *haematoxylin and eosin*. In the otherwise normal epithelium around the vesicle dyskeratotic necroses of some isolated cells are seen. These have dark pyknotic nuclei and bright red cytoplasm (primary keratinisation). They may also be shed into the lumen. *In the immediate vicinity of the vesicles there are sometimes some slightly enlarged finely vacuolar cells* (25) *Fig. 1*. In the lumen of the vesicles, ballooning cells are seen in addition to the dyskeratotic cells described above, some giant cells and cells with inclusion bodies. These cells all show more or less advanced necrobiosis: slowly they lose their nuclear staining properties and form a mass on the floor of the vesicle supported by the projecting papillae or regenerating epithelium. All necrobiotic cells described show a bright red cytoplasm. Towards the deeper layers, the staining slowly becomes weaker.

These processes can be better visualized when eosin stained preparations are observed under *the fluorescence microscope*. Normal epithelium does not show fluorescence. The horny layer and primarily keratinized dyskeratotic *cells show brilliant, golden-yellow* fluorescence. Only the cytoplasm shows fluorescence, while the nucleus remains dark. The necrotic cells in the roof of the vesicle and necrotic cells in the lumen of the vesicle show yellow-green to green fluorescence and clear transitional hues are seen. On the floor of the vesicle, the cell boundaries are still visible but the totally destroyed cells no longer show fluorescence.

Trichrome Staining

Using *Ladewig's* method, the horny material and primarily keratinising cells are stained a brilliant red. The dead cells have a faint grey-blue colour.

In the necrobiotic cells, all intermediate hues between red and blue are observed. The entire cell is either homogeneously stained or—especially in the giant cells—reddish-blue, blue and small wholly red parts alternate. The moderately large inclusion bodies do not stain at the cell membrane, an almost horny-red cytoplasm ring may form. Reddish, lilac, blue or grey cells lying on the bottom of the vesicle form a "*mosaic struct* " (25) also called "*piebald pictures*" (16) (Fig. 2) Using *Goldner's* method, a bright red colour is seen in the horny layer in the keratinizing cells and in the cytoplasm ring of the cells with inclusion bodies. The necrotic cells are of a faint greenish-grey colour In the necrobiotic, more or less homogenized, cells all transitions between red and greenish-grey are seen. *If trichrome staining is used any fl orescence is not seen.*

The findings after *fibrin staining* are as follows Using *Weigert's* method, horny material is strongly positive, in addition to the occasionally present fibrin. Staining—sometimes rather weak—is also seen in the dyskeratotic small cells. The other necrobiotic cells do not take up the dye. Using *Kockel's* method fibrin, the horn and all damaged cells are *stained pitch-black*. *Using Heidenhain's* method, only horn and fibrin are stained black. The necrobiotic cells are stained only dark or faintly grey

Fluorochromes. In preparations stained with *acridin orange* the horn and primarily keratinizing cells show a bright, golden-yellow fluorescence. Necrobiotic portions of the roof of the vesicle the protoplasm of the large ballooning cells in the lumen and a portion of the keratinizing cells that have already lost their nuclear staining show yellowish-green or green fluorescence and all intermediate hues. Inclusion bodies show red fluorescence when they become larger The halo does not display fluorescence. Occasionally yellow-green fluorescence and golden yellow droplets are seen in its substance. Towards the bottom, a very clear mosaic structure is formed, showing golden yellow greenish-yellow and green fluorescence of the cells (Fig. 3)

Methods for mucopolysaccharides and glycoproteids the PAS action is + or + + in most necrotic cells and enhances the autofluorescence of unstained sections. *Congo* red staining was always negative. In most cases, *thionine methylgreen pyronine* and *Taft's* method did not lead to appreciable staining, but enhanced the autofluorescence.

After *hodam e B staining* the horny material and dyskeratotic cells show very bright somewhat reddish silver-white fluorescence, whereas the necrobiotic cells in the roof of the esicle and lumen show a greenish-yellow to green fluorescence. In the dead, decomposing cells, the fluorescence disappears. It is a remarkable finding in the larger cells which after staining according to *Ladewig's* method contain a red ring below the cell membrane that a yellowish fluorescence is observed at the same location. This ring then dissolves in the cytoplasm which shows green fluorescence. In preparations stained according to *Ladewig's* method the red ring disappears in the blue-red cytoplasm.

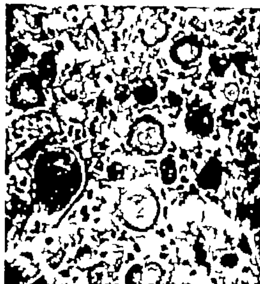


Fig 2 Necrobiotic cells in Herpes zoster vesicle presenting intermediate hues between red and blue, forming "piebald" pictures. Ladewig's stain, × 200.



Fig 3 Necrobiotic cells in Herpes zoster esicle. Piebald" pictures presenting golden-yellow—greenish-yellow and green fluorescence are formed. Acridine-orange stain, × 200.

Group I also contains some cases of pemphigus vulgaris, foliaceus and a case of pemphigus vegetans.

The most conspicuous symptom of this disease is acantholysis which is often associated with dyskeratosis. Necrosis of isolated cells is seen in the epidermis close to the vesicle and also in cells that have become detached and float in the contents of the vesicle. Using eosin, these stain bright red. By way of fluorescence microscopy the cytoplasm is seen to produce a reddish-yellow fluorescence, just as the normal horny layer. A portion of the akantholytic cells becomes enlarged (Tzanck cells) they are subject to necrobiosis and float to the bottom. The cytoplasm of the necrobiotic cells shows greenish-yellow fluorescence and green fluorescence when the nucleus does no longer stain. When total necrosis has occurred, the fluorescence disappears. The results of trichrome staining resemble those obtained in cases of herpes zoster. In the necrobiotic cells, intermediate hues between red and blue (Ladewig) or red and green (Goldner) are observed. Red horny inclusions are seen in the larger Tzanck cells which dissolve later. Staining for horn and fibrin shows results as those obtained in the herpes zoster group. In the intraepithelial micro-abscesses developing in the case of pemphigus vegetans, some large, detached, epithelial cells are seen between the leucocytes. Using eosin, these show yellowish green fluorescence.

Group II

This group contains cases of erythema multiforme, toxicoderma, drug exanthema. Some of these were primary cases, but some patients had experienced similar episodes in the course of several years and the latest episode had occurred 3–7 days before the present trial. There were no appreciable differences between cases with and without vesicles.

Not infrequently a more or less extensive necrotic process is seen in the swollen oedematous epithelial layer or in the epithelial roof of the vesicles in cases where subepidermal vesicles develop. In the immediate vicinity of the necroses or vesicles, some slightly enlarged non-necrotic cells with *finely vacuolar cytoplasm* are seen (25). The necrotic cells or larger coherent cell groups show more or less intensive red staining with *haematoxylin and eosin* (Fig. 4) and show a distinct greenish-yellow or green fluorescence in contrast to the bright golden yellow colour of the normal horny layer. In the preparations stained according to *Ladewig* method, the marked brilliant red staining of the normal horn and small dyskeratotic cells is see

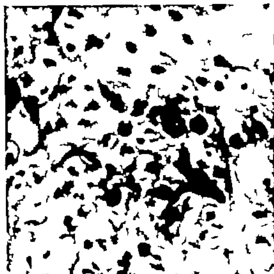


Fig. 4. Erythema multiforme. Necrotic cells in the epithel layer presenting intensive red staining. Haematoxylin-eosin, × 400

The homogenised necrotic epithelial cells assume a blue-red colour. *Goldner's* stain and *fibrin staining* yielded the same results as in group I. Using *fl orochrom* the same hues as those in group I are seen.

The finely vacuolar cells mentioned above show the same fluorescence as normal epithelium. *In fact these cells are damaged but still viable.*

On the basis of the results obtained in these studies it may thus be concluded that, under certain circumstances, a special "eosinophilic" necrosis may occur in the epithelial cells of the skin which morphologically corresponds to the fibrinoid necrosis of the connective tissue, vascular walls and of the liver cells mentioned previously (25). It appears that, in this necrosis of the skin, another substance keratin plays a part, on account of its great tendency to keratinization. Thus, fibrinoid, keratin and degradation products enter apparently into close relationships, thereby producing the described staining properties. These compounds can be called *"kerato-fibrinoid"*

Group III

This group includes cases of *Lyell's toxic epidermal necrolysis* and cases of *pityriasis lichenoides acuta* (*Mucha-Habermann*)

The best description of Lyell's disease is that recently published by *Braun Falco* (4) who stressed the occurrence of eosinophil necroses of the type of coagulation- and colliquation necrosis in the decomposing epithelia. Reference was also made to the hydropic and ballooning degeneration of the epithelia formation of vesicles, acantholysis etc.

The changes observed in our material were approximately the same as those observed in the first two groups. The coherent necrotic portions of the epithelia *show greenish-yellow fluorescence with* eosin, the smaller dyskeratotic cells show golden yellow fluorescence. The enlarged hydropic cells in the vesicles show yellow-green to green fluorescence. Using *trichrome stain g*, the latter display the intermediate hues described earlier If *fluorochromes* are used the changes are also the same as those in groups I and II. In the cases of Mucha-Habermann disease, the changes are less marked, probably on ccount of the extensive inflammatory changes. In general, the dyskeratotic alterations predominate in several cases, the necrotic epithelium shows the same intermediat hues after trichrome staining and in Wood's light as those described in the other groups. *The intermediate hues correspond t t ransitions between dyskeratosis fibrinoid necrosis and total cell destruction.*

Group IV

In this group the skin of patients who for diagnostic reasons were tested with ultraviolet light to determine their actinic sensitivity was studied. Development of the method and its evaluation were carried out by *Thyresson & Wennersten* (27) who also kindly supplied the histological preparations. Biopsies were collected after various intervals of irradiation.

After ultraviolet irradiation, a poor nutrition of the epithelium develop, the protein and lipoid reserves of the keratinocytes decrease The circulation is apparently also damaged. In several cases in this group *the finely vacuolar cells described* above were also found. A detailed description of the histological changes is outside the scope of the present article.

First, dyskeratosis of isolated cells or cell groups is seen. This is followed by the formation of small vesicles which later increase in size and contain pyknotic epithelial cells producing reddish-yellow fluorescence after *eosin staining*. In the necroses and the lumina of the vesicles, the primarily keratinizing cells are seen and also larger swollen cells, mostly without staining of the nuclei, which show all the staining and fluorescence reactions of fibrinoid necrosis. *Also rhodamin B staining* in cases of rather extensive necrosis produces a yellow-green fluorescence of the cells with fibrinoid necrosis, in addition to the golden-yellow fluorescence of the horny layer and dyskeratotic cells.

All other results of the various staining methods correspond to those applying to the first three groups.

Group V

This group includes the *controls* where routine staining shows distinct keratinization in the epithelium, but no reliable evidence of fibrinoid necrosis. The behaviour of pure fibrin in inflammations and of the fibrinoid in the vascular wall in cases of collagenosis (periarteritis nodosa) will be discussed later

The most simple changes are seen in *keratinizing squamous c ll carcinoma*. Such tumours of the skin the lungs and pancreas, the latter presenting extensive squamous metaplasia, were studied. It applies to these cases that fibrinoid necroses are either completely absent, or they are seen only indistinctly *in some enlarged cells at the* junction of the epithelial pearls and the tumour True intermediate hues are rarely seen.

Cases of *Darier's disease* with dyskeratoses, corps ronds" and grains keratinization predominates also in these cases. The grains are composed of a mass of eosinophil shrunken cells with pyknotic nuclei which show all reactions of keratin. The "corps ronds show no fluorescence as long as the nuclear staining is maintained afterwards they show the fluorescence of keratinized cells. It is only among the isolated larger akantholytic cells in the vesicles that cells may display fluorescence, as in the cells with fibrinoid necrosis. The intermediate hues to be obtained by trichrome staining are absent, however

The study also includes cases of *molluscum contagiosum and epithelioma calcificans Malherbe (pilomatricoma)* Any typical fibrinoid necroses of the decomposing cells were not observed in these cases only the well known signs of keratinization.

Fibrin controls included cases of pericarditis and endocarditi fibrinosa, fibrinous pneumonia and skin ulcers with fibrinous coatings. Fibrin always stains a bright red whether *eosi* or *Ladewig's* and *Goldner s* method are used It shows yellow to reddish-yellow fluorescence if *eosin acridin orange*

Taft's method or *rhodamin B* are used. *Weigert's* and *Kockel's* fibrin staining is always positive, *Heidenhain's* method is negative. The intermediate lines of fibrinoid necrosis are never seen.

Finally some cases of *fibrinoid necrosis* of the vessels in patients with periarteritis nodosa were studied. The material consisted of biopsies of the skin and tonsils, the autopsy material comprised specimens of the liver and kidneys.

Using eosin and Taft's method, the affected vessels are stained a faint red, presenting a strong fluorescence in Wood light. *Ladewig's* and *Goldner's* methods were positive in all cases.

Fibrin staining according Weigert' method was negative but positive if *Kockel's* and *Heidenhain's* methods were used.

A distinct *congophilic angiopathy* (23) (Fig. 5) was observed in 5 out of 9 cases. It was especially marked in the skin and tonsils. The congophilic material was never birefringent, only an increased reddish autofluorescence could be observed. A golden yellow and sometimes slightly greenish fluorescence was seen if *acridin orange* were used. *thioflavin* produced a very intense blue-white fluorescence in the affected vessels. It is remarkable that rhodamin B staining also showed an intense reddish-yellow or reddish-white keratin-like fluorescence, *especially in the skin* but also in the affected vessels of the other organs (Fig. 6).



Fig. 5. Case of periarteritis nodosa. Tonsillary artery with fibrinoid necrosis and congophilic angiopathy. Congo-red staining, × 125.

DISCUSSION

These and earlier studies (25) show that a fibrinoid necrosis may occur under certain circumstances, not only in the connective tissue but also in certain organs, e.g. in the parenchymal cells of the liver and epithelial cells of the skin.

The general causes of this necrosis include toxic and septic conditions as well as infections and circulatory disturbances. Among the latter, hypoxia anoxia appear to have a special significance (3, 10, 25) resulting in the characteristic epithelial cell changes in the form of a *finely vacuolar cytoplasm* in the immediate vicinity of necrobiotic foci (e.g. in erythema multiforme, Lyell's disease). The hypoxia may lead to vascular spasms, a tendency towards oedema and to vacuolar expansion of the endoplasmic reticulum. The injured cells lose apparently their ability to eliminate water (Ashworth) (2). *Pribram* & *Upham* (cit. 25) called this condition "*urticaria of the cells*". The hypoxia may also contribute to the development of dyskeratosis



Fig. 6. Case of periarteritis nodosa of the skin. Punch biopsy. Fibrinoid necrosis. Intensive fluorescence after Rhodamine B staining, × 200.

is proposed that the name "keratofibrinoid" be applied to this substance. Thus the concept of "fibrinoid necrosis" would obtain a more general significance in pathology

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SPINDLE CELL LIPOMA

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Angervall, L., Dahl, I., Kindblom, L.-G. & Säve-Söderbergh, J. Spindle cell lipoma. Acta path. microbiol. scand. Sect. A, 84 477–487 1976.

A clinical, light and electronmicroscopic study of 14 patients with spindle cell lipoma is presented. Spindle cell lipoma is considered to be a distinctive lipomatous tumour histologically characterised by a mixture of fat cells and fibroblast-like spindle cells, ultrastructurally similar to fibroblasts, in a matrix with varying amounts of collagen and mucosubstances. The tumours showed predominance for elderly men and all but one were situated in the posterior neck, shoulder region or upper back. The tumours varied between 1 and 9 cm, with a median value of 5 cm, in the widest diameter and were entirely or almost entirely situated in the subcutaneous tissue. A follow-up study of 11 patients, observed for 1–25 years, confirmed that the clinical course is benign. The differential diagnosis is discussed and it is emphasized that spindle cell lipoma is easily misinterpreted as sarcoma. Three tumours showed a pronounced nuclear polymorphism without mitotic activity thought to be regressive in nature. The cellular changes in these three tumours are presumed to be analogous with those in so called ancient neurilemmoma and therefore the name ancient spindle cell lipoma is proposed for the polymorphic spindle cell lipomas.

Key words: Spindle cell lipoma; lipoma; soft tissue tumour; pseudosarcomatous lesion.

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Spindle cell lipoma is a recently described type of soft tissue tumour characterized histologically by a mixture of fat cells and fibroblast like spindle cells in a matrix of collagen and mucoid material. This lipomatous tumour has frequently been misinterpreted as liposarcoma and other soft tissue sarcomas (*Enzinger & Harvey* 1975). Experience, however, of 114 cases presented by Enzinger and Harvey has shown the tumour to be perfectly benign occurring predominantly in the shoulder-neck area of elderly males.

This paper presents a clinico-pathological study of 14 patients with spindle cell lipoma including an ultrastructural study of formalin-fixed tumour tissue in 3 cases. Three of the tumours showed pronounced nuclear polymorphism without mitotic activity presumed to be analogous with the nuclear changes in so called ancient neurilemmoma.

MATERIAL AND METHODS

Two out of 14 cases were obtained from a Swedish series comprising some 800 cases of malignant soft tissue tumours reported to the Swedish Cancer Registry during a 6-year-period (1958–1963). Three cases were selected after reviewing various soft tissue lesions recorded in the Department of Pathology, Sahlgren's Hospital, Göteborg. Four tumours were sent to us for consultations from other pathological laboratories in Sweden. The additional 5 cases, examined in the period from 1972 and onwards, were diagnosed as spindle cell lipoma. Dr. Enzinger, chief of the soft tissue division,

TABLE 1 *Clinical Features in 14 Patients with Spindle Cell Lipoma*

Case no.	Age (years)	Sex	Anatomical distribution	Size (cm)	Follow-up period (years)	Recurrence
1	76	M	Posterior neck	6×4×2.5	4	None
2	50	M	Posterior neck	5×4	25	None
3	63	M	Upper back	3×3	4	None
4	67	M	Shoulder right	5×5	2	None
5	43	F	Posterior neck	2×1.5	8	None
6	44	M	Posterior neck	9×8	2	None
7	24	F	Right thigh, medial part	8×5×3	12	None
8	59	M	Shoulder right	8×5×2.5	1	None
9	51	M	Shoulder right	5×4	12	None
10	67	M	Shoulder right	2.5×2.5	2	None
11	65	M	Posterior neck	2×2	12	None
12	31	M	Forehead	2×2	<1	None
13	74	M	Posterior neck	1.2×1	<1	None
14	61	M	Posterior neck	3.5×2.5	<1	None

Armed Forces Institute of Pathology Washington, D C was consulted in the first of these cases and thereby drew our attention to this tumour entity later described by *Enzinger & Harvey* (1975)

The operative specimens were fixed in 4 per cent formaldehyde solution and embedded in paraffin. Five micron thick sections were stained according to the haematoxylin-van Gieson method and with haematoxylin and eosin. Gordon's and Sweet's silver impregnation was used for the demonstration of reticulin fibres, and Weigert's elastin method for studying elastic tissue in tumour vessels.

Alcian blue (Chroma-Gesellschaft) and toluidine blue stains were used at 2 different pHs, pH 2.5 and 0.5 and 4 0 and 0 5 respectively for the examination of glucose-aminoglycans (nomenclature according to *Jeanlos* 1960) as described previously (*Angervall et al.* 1973 *Kindblom & Angervall* 1975) These stains were performed with and without prior treatment of the sections with testicular hyaluronidase (hyaluronidase from bovine testes, type IV Sigma) (*Lapp & Steward* 1965)

Staining was performed according to *Scott & Dorling* (1965) at pH 5 6 with 0.05 per cent Alcian blue in 0 025 M acetate buffer with the addition of increasing concentrations of $MgCl_2$ in order to determine the "critical electrolyte concentration (CEC) of the dye-polymer binding in the mucoid material. The following series of $MgCl_2$ concentrations was used 0 0 M 0.025 M, 0 05 M, 0 1 M 0.25 M, 0.35 M 0 45 M 0 55 M 0.65 M 0 75 M 0.85 M and 1.0 M Staining time was 16 hours.

In 3 cases, small pieces of tumour tissue were embedded in glycol metachrylate and 1–2 micron thick sections were stained according to the haematoxylin-van Gieson method and with haematoxylin and eosin.

For electron microscopy about 1 mm small pieces of formalin-fixed tumour tissue from 3 patients were washed in 0 1 M sodium cacodylate buffer for 24 hours, fixed for 3 hours in ice-cold 1 per cent osmium tetroxide in cacodylate buffer pH 7 2, dehydrated in ethanol, embedded in Epon 812 and cut in a LKB Ultrotome III One micron thick sections were stained with toluidine blue. Ultrathin silver to grey sections placed on copper grids were stained by uranyl acetate and lead citrate, examined and photographed in a Philips EM 200 electron microscope.

In all cases, the clinical records were reviewed and the pertinent clinical and laboratory data were recorded. Follow up information was available in 11 cases.

Fig 1 Slices of a spindle cell lipoma showing grey-white, partly gelatinous, glistening, semitransparent cut-surfaces. Case 10, × 2 5

Fig 2 Spindle cell lipoma showing a mixture of fat cells and collagen. Case 3 H&E × 3.

Fig 3 Spindle cell lipoma showing lipoma-like areas blending with areas rich in collagen. Case 3, H&E × 4

Fig 4 A partly lipoma-like lobulated spindle cell lipoma poorly demarcated from the subcutaneous fat tissue. Case 1 H&E × 6.5


1


2


3


4

RESULTS

Information concerning age and sex of the patients, anatomic distribution, sizes of the tumours as well as follow up information is summarized in Table 1

The youngest patient was 24 years of age, the oldest 76 years. The median age was 61 years. Twelve of the 14 patients were men.

All the tumours were reported to have been slowly growing for months or years causing no pain, only slight discomfort or irritation.

In 4 out of the 5 retrospectively collected cases, the primary histological diagnosis was sarcoma. Two tumours had earlier been diagnosed as liposarcoma, one as myxosarcoma and another as fibrosarcoma. The remaining fifth tumour was diagnosed as a locally malignant myxomatous soft tissue tumour

Gross Appearance

The size of the tumours is given in Table 1 The median widest diameter of the tumours was 5 cm. The tumours were oval or spherical (Figs. 1 & 2) and 6 of them were of a lobulated appearance (Figs. 3 & 4) Grossly 7 of the tumours appeared well-demarcated from the surrounding subcutaneous fat tissue. The other 7 tumours were poorly delimited. All tumours were entirely or almost entirely situated in the subcutaneous tissue. Two tumours abutted upon the skin and another tumour was partly attached to the underlying fascia. Four tumours involved the underlying skeletal muscle tissue. The cut surfaces were greyish-yellow soft and/or grey white and firm. Glistening, partly gelatinous, translucent areas were seen in 4 of the tumours (Fig 1)

Light Microscopic Appearance

All tumours were characterized by a variable mixture of small, slender elongated spindle cells and fibres and bundles of collagen mingling with univacuolated fat cells (Fig 5) in 5 tumours mucosubstances were abundant, giving tumour a myxomatous character (Fig 6)

In all but 3 tumours, the spindle cells were fairly uniform in size and showed only slight polymorphism. The bipolar pale-staining cytoplasm of these cells was poorly outlined and fusiform (Figs. 7 & 8) Single spindle cells showed one or two small vacuoles within the cytoplasm. The nuclei were oval-shaped and vesicular with one or occasionally two small nucleoli. In places the nuclei were larger hyperchromatic, triangular or stellate-shaped with small vacuoles.

Three of the tumours (cases 5 10 and 12) differed considerably in that they were more cellular and showed marked nuclear polymorphism (Figs. 9 10 & 11) Many of the cells in these tumours were multinucleated giant cells with hyperchromatic nuclei, some of which contained single or multiple vacuoles (Fig. 10) The cytoplasm of some of these large cells appeared foamy or vacuolated and there were large tumour cells revealing numerous small hyperchromatic nuclear fragments as seen in karyorrhexis. Mitotic figures were not observed in any tumour

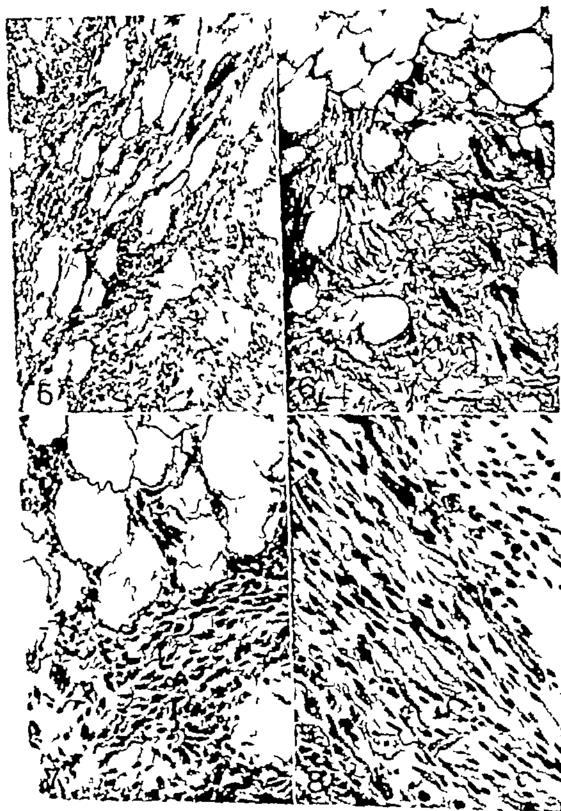
The number and distribution of spindle cells varied considerably both within different areas of the same tumour and from tumour to tumour The spindle cells were, for the most part, haphazardly distributed. In two tumours, the spindle cells showed a tendency to be arranged in parallel bundles (Figs.

Fig 5 Spindle cell lipoma showing a mixture of fat cells, spindle cells and haphazardly distributed bundles of collagen van Gieson × 90.

Fig 6 Haphazardly arranged elongated or stellate-like spindle-cells and collagen in a prominent myxomatous matrix with some univacuolated fat cells. H&E × 120.

Fig 7 A partly relatively cellular spindle cell lipoma showing univacuolated fat cells and spindle cells with uniform, elongated nuclei and bipolar tapered pale staining cytoplasm. van Gieson × 250.

Fig 8 Uniform elongated spindle cells showing a tendency to parallel arrangement and collagen fibres. H&E × 250



7 & 8) In one tumour the spindle cells were closely situated around small vessels.

The adipose tissue component of the tumours was prominent in all but 5 tumours, in 2 of which the lipomatous character was obscured by the numerous spindle cells, in the other 3 by the conspicuous collagen and abundant mucosubstances. The fat cells were univacuolated, mostly uniform in size with small peripheral nuclei (Figs. 5 & 7) In places the fat cells were more polymorphic but showed no atypicality of the nuclei Mingling with these univacuolated fat cells were multivacuolated fat cells, some of which possessed large and somewhat hyperchromatic nuclei However no multivacuolated lipoblasts were encountered.

The collagen was haphazardly distributed throughout the tumours, either as isolated delicate fibres in close proximity to the spindle cells or as homogeneous or fibrillar bundles of varying calibre. The collagen was abundant and formed the predominant component of 2 tumours and was more delicate and inconspicuous in 6 tumours In 2 tumours, there were irregular areas of homogeneous, hyalin material.

Five tumours were of a conspicuos myxomatous character (Figs. 6 & 12) with mucosubstances which stained positively with Alcian blue at pH 2.5 and metachromatically with toluidine blue at pH 4 0 Digestions with testicular hyaluronidase or lowering of the pH to 0.5 abolished these staining reactions thus indicating the presence of hyaluronic acid and the absence of sulphated glucose aminoglycans. Using staining with Alcian blue according to the Scott technique, the staining of the mucosubstance was visible up to 0 1 MgCl also indicating the presence of hyaluronic acid. In all these myxomatous tumours, there were numerous mast cells in the mucoid matrix.

Most of the tumours were poor in vessels, particularly in areas of a lipomatous character However the myxomatous tumours showed numerous, small capillary like, vessels measuring 5–20 microns in diameter These vessels frequently run an irregular branching course giving a plexiform vascular pattern (Fig 12) Some tumours also showed wider vessels up to 2 mm in diameter composed of an endothelial layer enclosed by dense fibrous tissue. There was no distinct elastic tissue, nor smooth muscle tissue in the vessel walls. An organizing thrombus was found in one of these vessels. Two of the tumours were more or less divided into separate lobules by irregular vascular spaces of this type.

The tumours were completely or predominantly situated subcutaneously and appeared fairly well circumscribed. However there was no distinct encapsulation Four of the tumours extended through the muscle fascia, down into the underlying muscle which was infiltrated and fragmented by the tumours and showed atrophic muscle fibres and proliferation of the sarcolemma (Fig. 11)

Electron Microscopic Appearance

Tumour tissue which had been preserved specifically for electron microscopy was not available in any of the cases. In 3 patients, however formalin fixed tumour tissue was washed in a buffer and postfixed in osmium tetroxide. The preservation was poor in 2 of the tumours, but in one tumour cellular details were relatively well preserved The tumour tissue in all 3 studied tumours revealed spindle shaped cells frequently arranged in parellel merging with abundant collagen fibre

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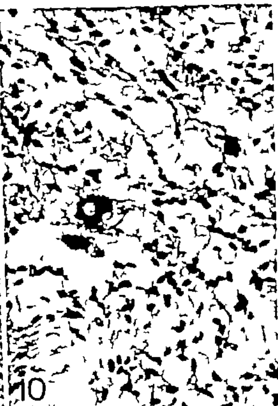
Fig 9 Spindle cell lipoma with haphazardly arranged spindle cells, some of which show large polymorphous, dark staining nuclei. H&E × 100

Fig 10 Higher magnification of the tumour illustrated in Fig 9 (case 10) showing monomorphous spindle cells and some large irregular cells with dark nuclei, one of which is vacuolated. H&E × 250.

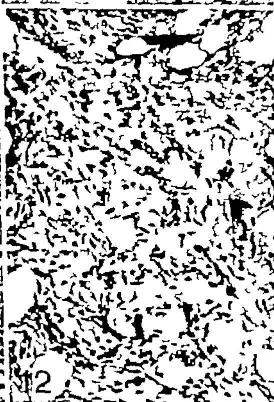
Fig 11 The deep portion of the tumour in Figs. 9 and 10 growing within striated muscle. H&E × 90

Fig. 12 A highly vascular area of a myxoid part of spindle cell lipoma showing small capillary-like vessels arranged in a plexiform pattern. H&E × 120.


9


10


11


12

13





bundles and large vacuoles such as those formed by fat cells. The tumour cells were rather regular in form most cells disclosed very elongated cytoplasmic outlines and centrally located nuclei (Fig 13) The nuclei were rather large and usually fusiform or oval, most of them with fairly smooth outlines showing small invaginations. The chromatin was finely dispersed with heterochromatin in small irregular clumps. The nuclei contained one single small nucleolus. The cytoplasm contained a moderate amount of organelles. Rough endoplasmic reticulum was rather abundant, mostly in the form of parallel membranes or small cisternae. The Golgi apparatus was recognizable in many cells in some it was rather prominent. Few mitochondria were demonstrated, but among these many showed signs of swelling, possibly an artefact due to fixation. Tiny uncharacteristic, loosely arranged, intracellular fibrils were encountered in some cells. A few tumour cells contained multiple droplets of lipid appearance (Fig. 14) No basement membrane-like material was found next to tumour cells. The collagen fibres showed characteristic cross-banding

Some cells in case 10 disclosed, however irregularly-formed nuclei where the nuclear membrane displayed deep folds and projections giving the impression of segmented or multiple nuclei (Fig. 15) These cells correspond to the polymorphic cells seen at light microscopic examination in cases 5 10 and 12. Some mast cells with characteristic, large, coarse granules merged with the tumour cells.

Fig 13 Spindle shaped tumour cell and collagen fibres (mainly in the upper part of the figure) The nucleus i fusiform with small invaginations. A prominent Golgi apparatus is seen to the left of the nucleus. × 8700

Fig 14 Multiple osmiophilic droplets of lipid appearance within tumour cell. × 3500.

Fig 15 A polymorphous tumour cell nucleus with deep folds. The chromatin shows a tendency of condensation towards the nuclear membrane. × 8000

Treatment and Follow-Up

All the 14 spindle cell lipomas were removed by simple excision. In all but 2 patients the tumours were excised with some margin or uninvolved surrounding tissue. On these 2 patients, wide surgical excision was performed within one month because the original diagnoses were liposarcoma and fibrosarcoma. No remnants of tumour tissue could be found in either of them. Follow up information was available in 11 patients whose follow-up ranged between 1 and 25 years with a median of 4 years. The additional 3 patients have been followed for less than one year All patients are living and well and recurrences or metastases have not appeared in any of these.

DISCUSSION

Spindle cell lipoma appears to the authors to be a distinctive soft tissue tumour of a fairly characteristic histological appearance. However there are, as in the present series, variations in cellularity in the amount and distribution of spindle cells, fat cells, and collagen both within different areas of the same tumour and from tumour to tumour It is imperative to recognize all the microscopic variations in this type of tumour because it may otherwise easily be misinterpreted as a soft tissue sarcoma as was the case in 4 out of the 5 retrospectively examined cases

Two tumours were originally diagnosed as liposarcomas. Highly differentiated fibrosing liposarcoma is composed of a mixture of fat cells and collagen. In this type of liposarcoma, however there is usually a more pronounced cellular and nuclear polymorphism than in spindle cell lipomas and multivacuolated lipoblasts are present (*Enzinger & Winslow* 1962, *Angervall et al.* 1975) Moreover the distribution of the collagen bundles in the spindle cell lipomas is characteristically irregular

Spindle cell lipomas of a prominent myxomatous character are clearly distinguishable from myxoid liposarcomas by the lack of multivacuolated lipoblasts and pooling of the

mucoid substance (*Enzinger & Winslow* 1962, *Kindblom et al.* 1975). It is of interest that, in some areas of the myxoid spindle cell lipomas in the present series, the vascular pattern was similar to the plexiform capillary pattern found in myxoid liposarcomas (*Enzinger & Winslow* 1962, *Kindblom et al.* 1975). Histochemical characterization of the mucosubstance does not help in the differential diagnosis since both tumours contain hyaluronic acid and lack sulphated glycosaminoglycans. Furthermore, unlike spindle cell lipomas, liposarcomas are usually more deeply situated and are larger in size (*Kindblom et al.* 1975).

Considering the difficulties involved in a differentiation between spindle cell lipoma and liposarcoma, it is interesting to note that we have seen 2 liposarcomas which histologically revealed areas indistinguishable from spindle cell lipoma. These 2 cases have been briefly described previously (*Kindblom et al.* 1975). One of the 2 liposarcomas also showed areas of a hibernoma like appearance both in the primary tumour and in the recurrence.

The diagnosis of myxofibrosarcoma was suggested for one tumour in the series. Myxofibrosarcoma may be difficult to differentiate from myxomatous spindle cell lipoma as seen in 3 tumours in the series because both tumours are often nodular, subcutaneously situated and occurring in elderly patients. However, myxofibrosarcoma is in general larger, more cellular with cellular and nuclear polymorphism and conspicuous mitotic activity. In addition, these tumours do not contain fat cells (*Merck et al.* to be published).

Three spindle cell lipomas in the series showed large vascular spaces, capillaries enclosed in dense, homogenous collagen tissue and areas predominantly composed of monomorphic spindle cells with a tendency to form parallel rows. In some respects such spindle cell lipomas may appear similar to neurilemmoma and benign hemangiopericytoma. However, there was no palisading of the nuclei or hyalin areas in any of the tumours in the present series as generally appears in neurilemmomas nor is there evidence ultrastructurally of cellular basement membranes and/or unmyelinated axons, as found in tumours of neural origin (*Fisher & Vuzevski* 1968). Benign haemangiopericytoma may in areas show myxoid changes and wide vascular channels. However, a characteristic pattern with ramifying sinusoidal or capillary-like vascular structures can be observed somewhere in these tumours (*Enzinger & Smith* 1976). Furthermore, the spindle cell lipomas contain unvacuolated fat cells which do not appear in neurilemmoma and haemangiopericytoma.

Three of the tumours (cases 5, 10 and 12), while possessing the same basic features as the remaining tumours in the series, showed prominent nuclear polymorphism and hyperchromasia. However, there was no mitotic activity and some of the nuclei showed vacuoles or signs of karyorrhexis suggesting regressive changes. Other apparently benign, soft tissue tumours such as ancient neurilemmoma (*Ackerman & Taylor* 1951) and myxoid polyps of the vagina described by *Norris & Taylor* (1966) show similar nuclear changes. Lesions demonstrating such changes are easily misinterpreted as malignant tumours. Ancient neurilemmomas may show some resemblance to these polymorphous spindle cell lipomas but the presence of fat cells and the distribution of collagen fibres and bundles in spindle cell lipomas help to differentiate these two tumours. We feel that ancient spindle cell lipoma would be an appropriate term for the polymorphous spindle cell lipoma with respect to its presumed analogy to ancient neurilemmoma.

Benign proliferative (pseudosarcomatous) lesions of soft tissue such as nodular fasciitis, which often has been confused with sarcoma (*Allen* 1972, *Dahl et al.* 197?), may in some cases or in some areas resemble spindle cell lipoma. However, the spindle-shaped cells occurring in nodular fasciitis are more slender, S-shaped or wavy, separating small clefts or slit-like spaces. Furthermore, areas with proliferation of capillaries, often radially arranged with extravasated erythrocytes as well as inflammatory cells are often encountered

in nodular fasciitis. The duration of tumour symptoms and anatomic location also may help in differentiating between these two lesions.

The ultrastructural appearance of the spindle cells was, in spite of the formalin fixation, sufficiently well preserved to appear similar to moderately differentiated mesenchymal cells in tendon, later differentiating into mature, more typical fibroblasts (*Greenlee & Ross* 1967). This similarity and the abundance of interstitial collagen supports the opinion held by *Enzinger & Harvey* (1975) that the tumour cells are fibroblasts or cells closely related to fibroblasts. Thus, the spindle cell lipoma is composed of fibroblast like cells and highly differentiated fat cells which possibly reflects the capacity of mesenchymal tumour cells to differentiate in different directions, i.e. cells with fat accumulation and collagen production. Our findings of spindle shaped tumour cells with lipid droplets lend support to such an opinion.

No recurrence was encountered in any patient in our series in spite of the fact that the surgical excision of the tumours in 2 patients might have been incomplete. The results conform with the findings obtained by *Enzinger & Harvey* (1975) viz. that the clinical course in patients with spindle cell lipoma is perfectly benign.

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GLYCOSAMINOGLYCANS OF CARTILAGE AND BONE TISSUE IN TWO CASES OF OSTEOGENESIS IMPERFECTA CONGENITA

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Engfeldt, B. & Hjerpe, A. Glycosaminoglycans of cartilage and bone tissue in two cases of osteogenesis imperfecta congenita. Acta path. microbiol. scand. Sect. A, 84 488–494 1976.

Epiphyseal cartilage and bone tissue from two cases of osteogenesis imperfecta congenita and one control case with similar skeletal age were examined regarding their glycosaminoglycan content. The diseased bone tissue showed a 3-fold increase in glycosaminoglycans, and 20–25 per cent of the chondroitin sulphate disaccharides seemed to be disulphated. The diseased cartilages showed only traces of material with characteristics of disulphated disaccharides. No disulphated disaccharides were isolated from the control materials. No difference between diseased and control material was indicated regarding molecular size, chondroitin-4-sulphate/chondroitin-6-sulphate ratio or contents of keratan sulphate and hyaluronic acid. The disease is thus associated with fundamental changes of the glycosaminoglycan structure and these changes may be of functional importance to the mineralization process as well as to the organisation of collagen.

Key words Bone cartilage glycosaminoglycans osteogenesis imperfecta

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Osteogenesis imperfecta is an inherited generalized connective tissue disease with unknown pathogenesis (1) The characteristic features with development of immature fragile bone tissue results in generalized osteopenia and multiple fractures. This status is often present already at birth, but sometimes the symptoms don t appear until later during childhood. Due to the time of onset, a congenital and a *tarda* f rm can be separated.

The disease has been associated with errors both of the formatio of collagen and of glycosaminoglycans ((\(s) Already in 1954 *Engfeldt et al.* (9) d nstrated an abnormal orientation of bone collagen in this disease and later studies (7 19) also indicated a disturbed collagen as well as GAG composition. In 1962 *Spencer* (?2) showed histochemical evidence of abnormal GAGs in the diseased bone and the analyses by *Solheim* (21) of isolated GAGs from a *tarda* case showed distinct changes of these compounds.

Solheim s study indicated the presence of a highly oversulphated chondroitin sulphate (CS) With the development of methods to study GAGs using chondroitinase digestion, it is now possible to verify and extend the findings of *Solheim* The aim of the present investigation is to study the GAC s of cartilage

and bone tissue in two cases of osteogenesis imperfecta congenita with special reference to the mode of sulphation.

MATERIAL AND METHODS

Long bones were taken at autopsy of two diseased children, who had died at birth (The authors are indepted to Dr *B. de Ruer*, Lund and Dr *I Gren berg* Hoddinge for obtaining the material) X-ray examination revealed multiple fractures and in both cases skeletal age corresponding to approx imately 6 months of gestation. Routine micro scopical examination verified the diagnosis.

Control material was obtained from a premature baby that had died from respiratory distress and that showed similar skeletal age but no signs of disease affecting the skeleton.

Tissue preparation. Bone tissue and cartilage were dried and defatted in several changes of acetone for 2 days at +4 C, and the dried tissues was milled in liquid nitrogen using a Spex Industries Freezer/Mill. The mineralised bone tissue was then recovered after centrifugation in an acetone-bromoform mixture with a density of 1 78 g/ml In the same way described earlier for the preparation of mineralized cartilage (12) Epiphyseal cartilage was dissected under a stereo microscope, avoiding admixture of adjacent bone tissue and the bone tissue was similary obtained avoding fracture ends and epiphyseal plates.

GAG preparation. The powder preparations were digested with papain and the GAGs were precipitated as described by *Hjertquist* (13) The precipitates were digested enzymatically as described by *Thunell* (23) to remove present nucleic acids. The GAGs were recovered from the digestion mixture by repeating the precipitation procedure (13) Part of the GAG preparation thus obtained was digested with chondroitinase as described by *Saito et al* (20)

Chromatographical procedures. Alliquotes of the GAG preparation corresponding to 25 μg of hexosamine were added to CPC microcolumns measuring 5 x 70 mm. To fractionate the GAGs according to molecular size and/or degree of sulphation, three of these columns were eluted with $MgCl_2$ solutions of increasing ionic strength according to *Antonopoulos et al.* (4) In order to separate different kind of GAGs, if present, three other CPC columns were eluted according to the n-propanol/methanol procedure described by *Anton poulos* & *Gardell* (3) One further column was eluted with 1 per cent w/ CPC, 0.3 M NaCl containing 0.05 per cent w/ CPC and 6 M HCl. The final fraction was hydrolysed as described below and the identity of the hexosamine was determined by ion exchange chromatography according to *Lohmander* (14) Keratan sulphate (KS) was isolated and determined chromatographically according to *Anseth et al.* (2)

Disaccharides obtained from chondroitinase digestion were subjected to ion exchange chromatography according to *Lohmander* (16) thereby separating disaccharides with different degree of sulphation, i.e. non- mono- and disulphated disaccharides.

Analytical procedures. Approximately 1 mg the exact weight determined on a Cahn electrobalance, was hydrolysed and the calcium contents of the hydrolysates were determined as described earlier (12)

Hexosamines were identified after 8 hours hydrolysis in 6 M HCl at 100 C and subsequent removal of the acid in vacuo over NaOH pellets. The Elson Morgan reaction was employed as described by *Antonopoulos* (4) Uronic acids were determined according to *Bitter & Muir* (6) N-acetyl hexosamine was assayed by a modification (18) of the Morgan Elson procedure. The 4,5-unsaturated uronic acids were determined according to *Hascall et al.* (11) The proportions of 4-sulphated to 6-sulphated chondroitin sulphate was calculated on monosulphated disaccharide fractions from the ratios of the Morgan Elson reactive material to 4,5-unsaturated uronic acids as described by *Lohmander* (16)

RESULTS AND DISCUSSION

The calcium contents of the bone preparations were very similar ranging from 20.9 to 21.6 per cent of the total dry weight. Assuming all calcium occurs as hydroxyapatite the organic dry weights were calculated

The recovery of uronic acids and GAG-bound hexosamines were found to be 3 times as high in the diseased bones as in the control bone tissue (Table 1) while the corresponding concentrations in the cartilage were similar (Table 2) The 3-fold increase in GAG content in the diseased bones over healthy ones is very significant in comparison with normal variations given by *Vejlens* (24) The high recovery figures for diseased bone tissue may partly be explained by admixture of mineralized cartilage in this immature bone tissue. It is, however less probable that these large values are entirely due to cartilage contamination. Firstly this would indicate that roughly 15 per cent of the tissue preparation would represent cartilage, regarding the GAG contents reported for mineralized epiphyseal

TABLE 1 *Recovery and Composition of GAGs from Bone Tissue*

	Uronic acids % org. dry weight	Hexosamines % org. dry weight	CS-4/CS-6 mol/mol
Osteogenesis imperfecta			
Case No. 1	0.60	0.38	3.3
Case No. 2	0.53	0.46	2.6
Control	0.20	0.16	2.7

* Calculated on monosulphated disaccharide fractions.

cartilage (17) Secondly the CS-4/CS-6 ratios *of diseased bone is similar to that of the con*trol material, also contradicting a major cartilage contamination. Thirdly traces of KS would probably have been detected if the bone tissue preparation contained a 5–10 per cent cartilage contamination. Fourthly the obtained histological sections did not contain significant amounts of mineralized cartilage. This fact, however does not exclude a minor admixture of cartilage in the bone tissue preparation, but if this was the case, the contamination must have been of marginal importance. It thus seems obvious that the diseased bone tissue contained increased amounts of GAGs, even if the exact figure can not be calculated on the present results.

On the other hand, *Solheim* (21) demonstrated in his study on a *tarda* case a GAG content that was lower than expected in the abnormal bone It might be that this divergence represents a difference between the two forms of the disease, i.e. a difference between the very old and very young bone in *osteogenesis imperfecta*.

The CPC cellulose chromatography (Figs. 1 and 2) show that slightly increased proportions of the diseased material were eluted with the 0.3 M NaCl fraction, in which fraction hyaluronic acid as well as low molecular weight chondroitin sulphate are supposed to elute. All the remaining material seemed to be galactosaminoglycans. The elution profiles (Fig 1) do not reveal significant changes of the molecular sizes of the GAG preparations. The results of the n-propanol/methanol elution procedure as well as analysis of chondroitinase digests indicate presence of chondroitin 4 and 6-sulphate the former isomer dominating (Table 1 and 2 Fig. 2) The 4-sulphate/6-sulphate ratio seems to be similar for the diseased and control bone tissues, which supports the opinion that the degree of development of the tissues were similar *Solheim* studying an adult case of the disease, demonstrated infrared characteristics of

TABLE 2 *Recovery and Composition of GAGs from Epiphyseal Cartilage*

	Uronic acids % dry weight	Hexosamines % dry weight	Keratan sulphate hexosamines % dry weight	CS-4/CS-6 mol/mol
Osteogenesis imperfecta				
Case No. 1	8.9	7.7	0.15	0.72
Case No. 2	8.8	7.9	0.19	0.61
Control	8.6	7.4	0.17	0.96

* Calculated on mono ted disaccharide fractions.

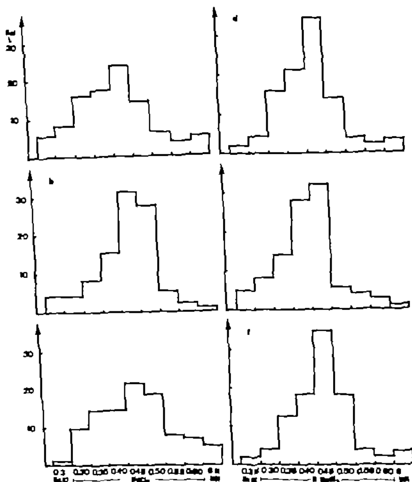


Fig 1 Elution profiles from CPC-cellulose microcolumns of glycosaminoglycans from bone (a–c) and cartilage (d–f) in osteogenesis imperfecta (a, b, d and e) and control material (c and f) The elution was performed with solutions of increasing ionic strength, separating the material according to molecular size and/or degree of sulphatation.

the 4- as well as of the 6-sulphated isomers of the isolated CS. This is remarkable, since adult bone is supposed to contain CS-4 only The CS-4/CS-6 ratios of cartilage GAGs were as expected for normal cartilage. Presence of dermatan sulphate in both tissues can t be excluded since traces of material remained in the column after elution with the acid $MgCl_2$ solution. Similarly small amounts of HS were obtained from the examined cartilages, while the bone tissue preparations showed no trace of this GAG

As shown in Fig 3 the GAGs of the diseased bones contained non mono- and disulphated disaccharides, while only non and monosulphated ones were found in the corresponding control material. In case of the diseased cartilage only traces of material were eluted similar to disulphated disaccharides. *Solheim's* finding of a 2 1 molar sulphate hexosamine ratio (21) could thus partly be confirmed since some of the disaccharide units were disulphated. This is very interesting, since oversulphated dermatan sulphate has been reported to occur in human aorta (10) but to our knowledge no such CS has been demonstrated in human tissues. It must be born in mind that the material we have

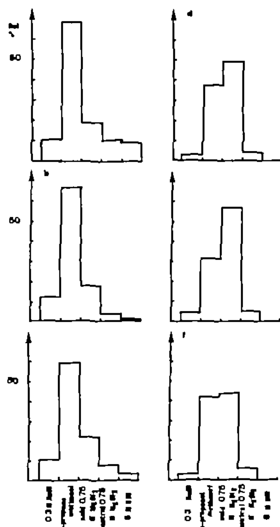


Fig 2 Relative distribution of the glycosaminoglycan-bound hexosamines from bone (a–c) and cartilage (d–f) in osteogenesis imperfecta (a, b, d and e) and control material (c and f) The CPC-cellulose microcolumns were eluted according to the n-propanol/methanol procedure.

studied is obtained at autopsy and thus there is always the risk of autolytic effects diminishing the amount of disulphated disaccharides. The present findings seem to indicate a disturbance in the GAG metabolism of the bone tissue in osteogenesis imperfecta The exact nature of this disturbance is however unclear

The recent results presented by *Cuervo et al* (8) and by *Lohmander & Hjerpe* (17) indicate that the strongly polyanionic GAGs in their macromolecular organization, as proteoglycans, participate in the mineralization process. A metabolic disturbance resulting in definitely increased amounts of this polyanion, and also in changes in the sulphation mode might disturb the enucleation of mineral embryos. Information is however lacking regarding the calcium binding qualities of these GAGs, and perhaps more important of the actual PGs.

The strength of the bone tissue is related to the organization of collagen fibres rather than to the mineral content (5) The high fracture frequency in this disease might partly be explained by the disarrangement of collagen fibres (9) and partly by the low rate of bone formation. The occurrence of disulphated disaccharides in the chondroitin sulphate might indicate an abnormal synthesis, and it might be that the collagen disturbance is secondary to the GAG abnormality It is known that GAGs and collagen react with each other and that this interaction is dependent on the negative charge of the GAGs (24) The basic information concerning the way GAGs and collagen interfere with each other is however still too scanty to allow safe conclusions in this respect. Studies on biopsy material would be preferential since autolytic effects could be excluded To do so the chromatographic procedure for separating disaccharides must be modified to allow studies of smaller samples. Such studies are in progress at our laboratory

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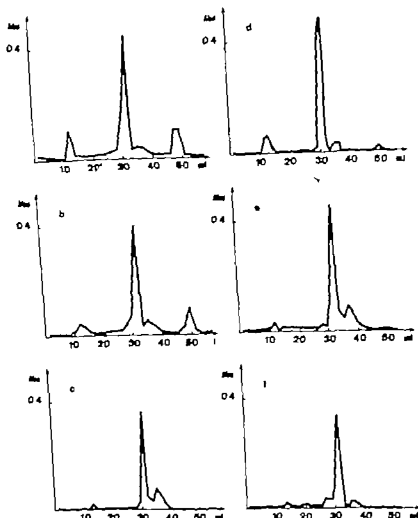


Fig 3 Carbazole reacting material obtained from chromatography on Aminex A-25 (100 × 3 mm) of chondroitinase digests from bone (a–c) and cartilage (d–f) Elution was performed with a linear LiCl gradient (0–3 M) of 60 ml. The upper and middle curves (a, b, d and e) show the profiles obtained from osteogenesis imperfecta and the lower curves (c and f) represent the control material. Nonsulphated disaccharide residues are eluted with approximately 12 ml monosulphated with 30–32 ml and disulphated with 48–50 ml of the gradient Note the distinct peak in the disulphated region of the chromatograms from the diseased bone preparation.

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THE LOCALIZATION OF PRECANCEROUS CHANGES AND CARCINOMA AFTER PREVIOUS GASTRIC OPERATION FOR BENIGN CONDITION

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Hammar E. The localization of precancerous changes and carcinoma after previous gastric operation for benign condition. Acta path. microbiol scand. Sect. A, 84 495–507 1976.

Sixty-five patients operated on for benign gastric condition showed at re-operation in 9 cases precancerous changes, in 22 cases carcinoma *in situ* and in 34 cases infiltrating carcinoma. The median time to elapse between the two operations was 20 years. The pathological diagnosis established at the first operation was most often benign duodenal ulcer. Investigation revealed in 9 cases precancerous changes and in 47 cases various grades of adenocarcinoma within the anastomosis. In 37 cases of Billroth II operations, 5 precancerous changes, 15 carcinomas *in situ* and 17 infiltrative cancers were located on the posterior gastric wall near the efferent intestinal loop. In these cases there were additional changes, one precancerous, 7 carcinoma *in situ* and two infiltrating cancers, situated against the cephalic part of the afferent intestinal loop. Nineteen Billroth II patients had precancers or cancers at other sites [illegible] anastomosis or gastric remnant. The remaining 9 cases of Billroth I, gastro-enterostomy [illegible] gastric Roux-en-Y procedures are too few to allow conclusions concerning the [illegible] precancer or cancer in the anastomosis.

Key words: Gastric operation precancerous changes carcinoma *in situ* [illegible] localization.

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Cancer of the gastric remnant in patients previously operated on for a benign ulcer was first seen to develop in a gastro-enterostomy (GE) in 1926 and was described by different authors *Beatson* (1926) *Owen* (1926) and *Schwers* (1926) *

In 1928 *Wolfsohn* maintained that cancer could not arise in a gastro-enterostomy because "dass im Dünndarm [illegible] mende Schutzstoffe [illegible] was not found in any [illegible] cases of gastro-entero[illegible] studies (*Braun* 1928 [illegible] *et al.* 1929 *Large* [illegible] *Hartman* 1937) [illegible] shown to arise [illegible] operation includin[illegible] tion, usually pep[illegible]

In 1930, *Ei*[illegible] primary cancer [illegible]

* *Schwers* quoted as *S hwers* by *La je* 1935 *Prus* 1938 and *Bandman* 1933

previous gastric resection with gastro-jejunostomy (Billroth II, B II) for benign ulcer. Subsequent studies (*Singer* 1932 and *Ransom* 1936) could verify this finding and in 1938, *Prin.* and *Konjetzny* showed primary cancer of the gastric remnant to be a special form of tumour.

In 1954 cancer in a gastroduodenostomy (Billroth I, B I) was first described by *Heinzel & Laqua*.

Kuhlmayer & Rokitansky claimed in 1954 that the fundus was the most common site of cancer (23 out of 40 cases). On the other hand, cancer within the cardia and anastomosis was rare. Cancer in the anastomosis was seen after B II in five cases and after GE in one case while cancer generally did not occur after B I.

The most common sites of tumours in the surgically treated stomach were defined in several studies: *Schreiber et al.* 1964 (B I or B II: entire stomach 8, *anastomosis* 5, fornix and cardia 3, unknown 2 cases); *Kootz* 1967 (GE: antrum 4, *anastomosis* 3, fundus and entire stomach 2 cases; B II: entire stomach 5, *anastomosis* 4, cardia 2, minor curvature 2, fundus, duodenal stump and unknown one case each) and *Kivelitz et al.* 1973 (*anastomosis* 18, fundic region 16, minor curvature 4 and cardia one case).

According to the literature, investigators are quite in agreement that carcinoma by and large begins in the anastomosis itself (*Freedman et al.* 1954, *Krause* 1957, *Boeckl et al.* 1963, *Kronberger et al.* 1968, *Kobayashi et al.* 1970, *Morgenstern et al.* 1973 and *Dahm et al.* 1975).

PROBLEM: Is there within the previously resected stomach a specific area which is particularly liable to cancerous changes?

This investigation deals with the problem whether or not cancer arises at specific sites in the anastomosis in relation to the efferent and afferent small intestinal loops and the posterior anterior gastric wall.

DEFINITIONS AND NOMENCLATURE

Stages of Changes

I *PRECANCEROUS* (preCa): moderate to marked epithelial atypia, probably not reactive within the mucosa (see Fig. 1, c.f. *Järvi et al.* 1951)

II *EARLY GASTRIC CARCINOMA* (*Murakami* 197). Synonyms: surface carcinoma of the stomach, intramucosal carcinoma, das Frühkarzinom (*Grundmann et al.* 1974 and *Hermanek* 1975)

a MUCOSAL CARCINOMA or *CARCINOMA IN SITU* (CaO): marked epithelial as well as glandular atypia or/and focal loss of glandular structures. The mucosal cancer does not penetrate into the muscularis mucosae (see Figs. 2 and 5)

b SUBMUCOSAL CARCINOMA: cancer situated within the mucosa and submucosa but not infiltrating muscularis propria (see discussion)

III *INFILTRATING CANCER* (InfiltCa) or advanced gastric carcinoma: infiltration of the muscularis propria (see Figs. 3 and 4)

Histological Types of Gastric Carcinoma

1 Intestinal type. (See Figs. 2 and 3)

2 Diffuse gastric type. (See Fig. 4) *Laurén* 1965.

Signet ring cell cancer (scirrhous carcinoma and linitis plastica) represent a special type of diffuse adenocarcinoma (see Fig. 5)

PATIENTS AND METHODS

The selected clinical series consisting of 65 patients was obtained as follows:

1) Twenty-three cases derived from goal-directed medical check-ups (*Huldt*) of 1,622 patients, all of whom had their first operation for a benign gastric condition, usually peptic ulcer at the hospi-

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Fig. 1 shows intestinal metaplasia and atypia-precancerous changes. H&E. × 110 (c.f. *Järvi et al.* 1951 and *Laurén* 1965)

Fig. 2 Gastric mucosa with marked glandular and epithelial atypia-carcinoma in situ (key case, Fig. 8). H&E × 100

Fig. 3 Gastric mucosa with adenocarcinoma of intestinal type (key case, Fig. 9). H&E × 100.

Fig. 4 Gastric mucosa with poorly differentiated adenocarcinoma of gastric type. H&E × 100.





Fig 5 Histological section of a gastro-jejunostomy (B II) showing extensive growth in the gastric mucosa of signet ring cell cancer (arrows) H&E. × 63 Inset × 250

tal in Lund during the period between 1930 and 1960

2) Forty-two patients surgically treated during the period between 1930 and 1969 who sought medical care at the hospital in Lund (*Haldt Liedberg* and *Eriksson*) or another hospital served by the same pathological department.

All of the patients (7 women and 58 men) were re-operated on during the period between 1969 and 1976. The patients are divided into three groups: precancerous (I) early gastric carcinoma (II) and infiltrating cancer (III)

It was possible in 35 cases to re-examine specimens from the primary operation. In the remaining 30 cases, all the clinical details and primary diagnoses were available.

The following investigative routine was applied immediately to the fresh specimens which were labelled with sutures by the surgeon in order to distinguish afferent and efferent limbs of the small intestine. The tissue was cleansed with saline at body temperature.

Billroth II specimens were processed according to Figs. 6 a–c (c.f. Figs. 8 and 9)

After cutting along the mesenteric border of the small intestine, a rectangular specimen with an eccentrically situated stoma was usually obtained, which could be viewed from either the gastric or intestinal surface. See Fig. 6 b.

The specimens were photographed from both sides (c.f. Figs. 8 b1 and b2)

In order to obtain the best orientation, the gastric cuff was opened, usually through the afferent loop, and the specimen was mounted on a

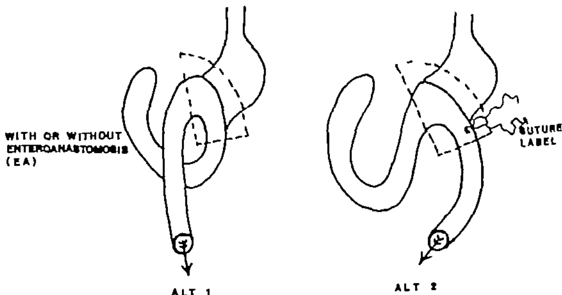


Fig 6 a. There are two types of B II operations one in which the efferent loop lies to the right (alternative 1) nd nother in which the loop lies to the left (alternath 2) Dotted line: surgically excised areas.

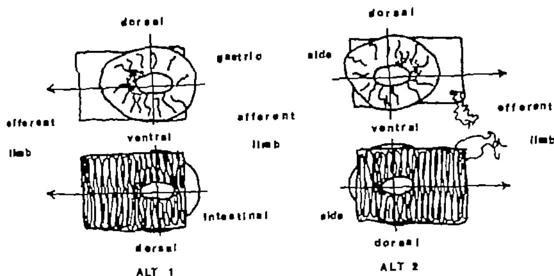


Fig 6 b shows specimens from B II operations which can be viewed from both the gastric and intestinal aspects after cutting open the small intestine along the mesenteric border

cork board and fixed in 10 per cent formaline according to Fig. 6 c (c.f. Fig. 8 c)

Gastro-enterostomy specimens were processed according to Figs. 7 a–b (c.f. Fig. 10)

The minor curvature of the stomach and the intestinal mesenteric border were cut open. See Fig. 7 b.

Specimens from the various types of operations (c.f. key cases Figs. 8–11) could be handled in principle, according to the presented schemes, whether or not the mucosa was macroscopically "normal" or contained a large infiltrating tumour mass.

Histology

In most cases in groups I and II, the entire anastomosis was histologically studied while in group III, various parts were selected.

Routine stains: Haematoxylin and erythrosin (H&E) supplemented in certain cases with van Gieson and PAS (according to Mac Manus)

RESULTS

The 65 patients were divided into three groups

I 9 re-operated cases with precancerous changes.

II 22 re-operated cases with early gastric carcinoma (carcinoma *in situ*)

III 34 re-operated cases with infiltrative cancers.

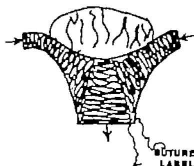
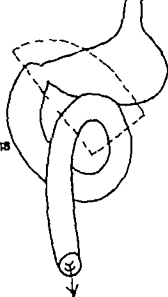


Fig 6 c shows the B II specimen opened through the afferent loop.

Re-examination of 35 gastric specimens obtained at the primary operation for ulcer disease showed no precancerous changes or cancers.

In groups I and II, 20 out of 26 patients had been given a Billroth II in which the efferent loop was situated on the left side (Fig 6 a, alternative 2) In group III this procedure had been used in 17 out of 30 cases among which 5 were tumours involving the entire gastric remnant and two were tumours situated in the cardiac region. In the remaining 19 cases it was not known which loop was efferent or afferent. Sixteen operative specimens from groups I and II together with 8 from group III were completely mapped



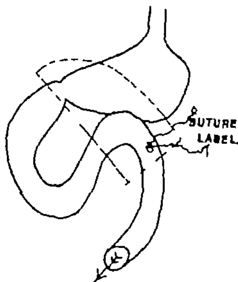


Fig 7 a shows GE after the two types of operations. Dotted line: surgically excised areas. (c.f. Fig. 6 a)



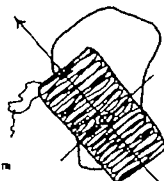


Fig 7 b shows GE specimen cut open.

out by way of photographs (c.f key cases *Figs. 8–11*) The site of the primary ulcer in patients in the three groups was in 31 cases the duodenum, in three cases the pylorus, and in 17 cases the stomach. In 14 cases the site of the ulcer is undefined. Mean age at first and second operation was 43.0 and 58.8 years (group I) 40.2 and 63.6 years (group II) and 46.9 and 67.7 y (group III) respectively In Table 1 the type of the first operation is correlated with the diagnosis at the second operation. Only two patients with Billroth II had an EA (male group I and female group II)

Classification of the cancer type according to Laurén showed 27 cases of the intestinal type and 29 cases of the diffuse gastric type Group II comprised two patients with signet

TABLE 1 *Type of First Operation and Diagnosis of Gastric Lesions at Second Operation on 65 Patients*

	B II		B I		GE		Roux-en-Y		
	F	M	F	M	F	M	F	M	
I precancerous		6	2					1	9
II carcinoma in situ	4	16				2			22
III infiltrative cancer	1	29		3		1			34
	5	51	2	3	0	3	0	1	65

ring cell cancer *in situ* (see Fig 5) Twenty patients within group III died of diffuse gastric cancer while 5 patients died of cancer of intestinal type.

All patients in groups I and II had changes located solely at the anastomosis.

Infiltrative cancer of the gastric remnant was in three cases (two B II and one B I) seen in the cardiac region, in 6 cases (5 B II and one B I) in the entire remnant and in 25 cases (23 B II one B I and one GE) adjacent to the anastomosis.

Two out of the 23 patients discovered in goal-directed medical check-ups had precancerous changes. Sixteen cases had carcinoma *in situ* The remaining 5 cases showed infiltrating cancers.

Site of changes in Billroth II operated patients

In group I, 5 out of 6 patients had precancerous changes situated adjacent to the efferent limb In only one of these cases, the efferent loop was on the right side. This specimen also showed diagonally situated changes. In the remaining case it was not known which loop was efferent or afferent.

In group II 7 out of 20 patients had diagonally situated carcinoma *in situ* (c.f Fig 8) In only one of these cases was the efferent loop on the right side In 8 cases, cancer was seen only at the efferent limb. The remaining 5 cases also had tumours at the anastomosis, though in these it was not known which loop was efferent or afferent.

In group III 2 out of 23 patients had diagonally situated cancers. Fifteen cases had cancer only at the efferent limb (c.f Fig. 9) In the remaining 6 cases it was not known which limb was efferent or afferent.

Thus, among 49 cases of B II with anastomosis it was possible to select 37 patients showing changes at the efferent loop extending mainly towards the posterior gastric wall. In 10 of these cases, additional changes were diagonally situated towards the afferent loop cephalically within the jejunum.

The group of patients with infiltrating cancer comprised also three cases of precancerous changes and 9 cases of carcinoma *in situ* in most cases, located in the neighbourhood of the main tumour mass.

In 4 out of 5 patients with Billroth I (see Table 1) the changes were situated within the anastomosis at the minor curvature. In the remaining case, a large tumour was seen in the cardia at the major curvature.

Finally three cases of GE and one of gastric Roux-en Y showed changes located at the anastomosis. Two cases of GE with carcinoma *in situ* showed cancers at the efferent limb extending towards the posterior gastric wall and, in one of these there was an additional tumour at the afferent loop

DISCUSSION

A multiplicity of investigations dealing with primary cancer in the gastric remnant in patients previously operated on for a benign condition has been published (review articles by *Freedman & Berne* 1954 *Boeckl & Lill* 1965 *Dahm & Werner* 1975 and monograph by *Dahm & Rehner* 1975)

Dahm & Werner reported in 1975 that

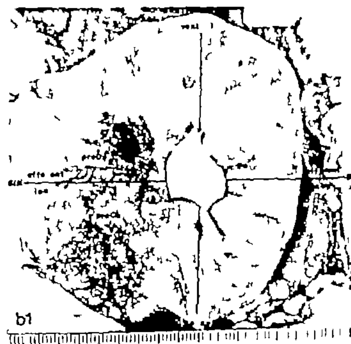


Fig 8 Key Case A 69-year-old man was in 1949 subjected to gastro-jejunostomy (B II) because of gastric ulcer Re-operated in 1975 on the indication of multifocal adenocarcinoma *in situ*.

a) Resected specimen where the efferent loop is labelled. The jejunum is opened along its mesenteric border

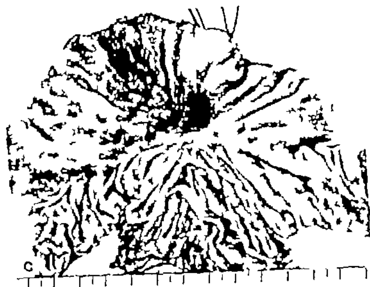
b) The specimen mounted, showing the irregular anastomosis from the stomach (b1) and the small intestine (b2) Diagonally situated carcinoma *in situ*

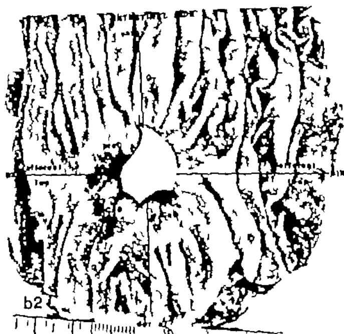
c) The same specimen presenting the opened anastomosis, mucosa on one side.



cancer in the gastric remnant was 4–7 times more common in men than in women. According to *Saegesser et al* (1972) this figure is 17–1 In the present study the corresponding figure is 10–1 in favour of men. In Japan the increase in frequency of anastomosis cancer is seen to be lower than that observed in other countries, in spite of the fact that gastric cancer in Japan is about 6 times more common than in the USA (*Dahm & Werner* 1975)

Nicholls (1974) reports the incidence of





anastomous cancer to be about one in 76 partial gastrectomies. Accordingly the resulting figure for the medical check up study ought to be 21 expected cancers as against the 23 cases which have already been discovered and re-operated on.

The primary indication for operation was predominantly gastric ulcer in according to the studies reported by *Helsingen et al.* (1956) *Boeckl et al.* (1963), *Kronberger et al.* (1968) and *Nicholls* (1974). Other investigations, e.g. by *Saegesser et al.* (1972)



Fig 9 Key Case A 68-year-old man was subjected to gastro-jejunostomy (B II) in 1944 because of pyloric ulcer Re-operation in 1975 indicated by the diagnosis gastric adenocarcinoma

a) Resected specimen where the afferent loop is labelled. A large polypoid tumour on the posterior gastric wall protruding into

b) the anastomosis at the efferent loop

Morgenstern et al (1973) in the present study however a predominance of duodenal ulcer was observed *Freedman et al.* (1954) *Stalsberg et al* (1971) among others, found no difference in the cancer incidence as related to the primary ulcer site

The time interval between operations covered 5–45 years, averaging 20 years (*Helsingen et al* 1956) which agrees with findings in the present investigation. Both sexes were approximately the same age at the time of the operations apart from two women in group I (preCa) who were several years younger than the men at the time of operation. The lowest mean age for both sexes at first operation was 40 years in group II (Ca0) and the highest at the second operation, 68 years, in group III (Infilt.Ca)

According to many surgeons, EA prevents bile reflux which is one of the symptoms of an incompetent sphincter Both cases with EA showed bile reflux clinically A great number of patients had reflux symptoms. No EA was seen in the group of patients with infiltrating cancer

It is quite difficult to handle a formalin-fixed stomach which has not been mounted on a cork board or labelled with sutures. To obtain the best results, fresh, suture-labelled specimens should be processed according to the methods described. Strictly speaking only 24 cases corresponded in all details to the investigative routine mentioned above.

The intestinal type of cancer (*Laurén* 1965) is more common in the group of patients with carcinoma *in situ* (20/27) In 9 cases of precancerous changes, intestinal metaplasia was very prominent (c.f *Jarvi et al* 1951) On the other hand, diffuse gastric carcinomas are very common in the group of patients with infiltrating cancer (27/29) The two cases of signet ring cell cancer *in situ* (see Fig 5) are apparently rare Surprisingly this study shows no early gastric cancer of the submucosal type

Most patients died of diffuse gastric cancer while the two patients with signet ring cell cancer *in situ* are still alive three years postoperatively and without signs of recurrence.

In the literature, the site of the cancer is stated to be in the stoma fundus, either curvature cardia or the entire gastric remnant. With few exceptions, information is scanty as regards the site of human anastomosis cancer relative to the position of the small intestinal afferent and efferent limbs and relative to the anterior and posterior gastric walls. *Morgenstern et al.* (1973 p. 33 demonstrated a "Typical specimen of car inoma of gastric stump. The lesion is seen arising from the afferent limb aspect of gastrojejunal stoma (c.f *Dahm & Rehner* p 31 Abb. 18, 1975)



Fig 10 Key Case A 78-year-old man treated surgically with gastro-enterostomy (GE) due to duodenal ulcer Re-operation in 1975 indicated by the diagnosis anastomotic ulcer and a strong clinical suspicion of cancer

a) Resected specimen where the afferent loop is labelled. Tight anastomosis within the pyloric canal with two separate polypoid changes (arrows) on the posterior wall towards the efferent loop Scarred narrowing of the pylorus, baggy dilatation along the major curvature. The polypoid gastric changes represent carcinoma *in sit*

b) The stoma as seen from the small intestine displays an ulceration (arrows) at the afferent loop towards the anterior wall.

In several other publications, the main tumour mass within a Billroth II anastomosis, as shown by diagrams and pictures, is situated within the stoma mainly on the posterior gastric wall near one of the intestinal loops (*Large* 1933 p 2307 *Prms* 1958 p 152, *Edelhoff* 1952 p. 149 *Pack et al.* 1958 p. 1029 *Berkowitz et al* 1959 p 695 and *Gerstenberg et al* 1965 p 2185)

In 1974 *Hammar* showed primary cancer within the anastomosis located on the posterior gastric wall near the efferent small intestinal loop In 1975 (*Hammar* 1975 and *Hammar et al* 1975) also additional changes were shown to be situated against the cephalic part of the afferent small intestinal loop.

Experimental studies of anastomosis carcinoma in the rat (*Dahm & Werner* 1973) has demonstrated the site of nitrosoguanidine induced cancer in resected stomachs. All Billroth II rats developed tumours within the gastric anastomosis, the tumours growing towards the minor curvature. In the Billroth I animals, cancer developed at the anastomosis and minor curvature in 80 per cent of the cases. The Billroth I cases presented here are too few to allow conclusions concerning the localizations of anastomosis cancer The precancerous changes were situated at the anastomosis and extended towards the minor curvature. In two cases of advanced cancers (c.f Fig 11) the main tumour mass also occupied this site (c.f *Saegesser et al* 1972)

Generally there are two types of Billroth II operations, one in which the efferent loop lies to the right and another in which the loop lies to the left. The latter type of operation is more frequently represented in the present study Information regarding these types of Billroth II operations are often lacking in the



Fig 11 Key Case A 67-year-old man on whom gastro-duodenostomy (B I) due to benign gastric ulcer had been performed in 1966 Re-operation in 1975 indicated by the diagnosis gastric carcinoma. The picture shows the whole stomach and an ulcerated anastomotic cancer invading the minor curvature. No metastasis. The immediate post-operative course was uneventful, but the patient died 5 days later from pulmonary embolism.

surgeon's report. The correct loop can be identified with certainty at re-operation whereupon this information is passed on to the pathologist by labelling of one of the loops.

The Billroth II and gastro-enterostomy specimens which were thoroughly studied are still too few to allow definite conclusions about a site for precancer and cancer extending towards the cephalic part of the jejunum in the afferent loop. On the other hand, this investigation presents strong evidence for a "typical" site of cancer growth within the Billroth II stoma extending towards the posterior wall near the efferent loop

The need for further investigation in this field is obvious, particularly with a view to determining more exactly the site of carcinogenesis in operated stomachs of various types, and the factors influencing its occurrence.

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GROWTH OF RABBIT AORTIC SMOOTH MUSCLE CELLS IN SERUM FROM PATIENTS WITH JUVENILE DIABETES

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Ledet, T Growth of rabbit aortic smooth muscle cells in serum from patients with juvenile diabetes. Acta path. microbiol. scand. Sect. A, 84 508–516 1976.

The effect of human diabetic serum on the growth of rabbit arterial smooth muscle cell cultures was studied in the stationary phase of growth. The serum was obtained from young, male, non-obese, juvenile diabetics and non-diabetics. The experiments were carried out using dialysed as well as non-dialysed serum. The concentration of cholesterol and triglycerides were equal in normal and diabetic serum. Media supplemented with diabetic serum from both short term and long term diabetics stimulated the outgrowth of the smooth muscle cells significantly ($2p<0.01$) A statistically significantly stimulation of growth was also observed using dialysed human diabetic serum ($^{2}p<0.05$) Autoradiographic studies showed that the number of ^{3}H thymidine labelled cells and of cells in mitosis increased appreciably after incubation in diabetic human serum ($2p<0.005$) The present data show that human serum from juvenile diabetics contains a factor or factors which promote an excessive growth of arterial medial cells. The factor(s) is not lipids as hyperlipemia was not present nor is t glucose, aminoacids, fructose or ketones, as the growth effect remained after dialysis

Key words Diabetic angiopathy atherosclerosis tissue culture growth factors.

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Disease in the heart and the large blood vessels occurs with increased frequency in clinically manifest diabetes mellitus (7 14 17) There is also an excess of mortality among diabetics with cardial infarction (7 22)

It has been thought that the lesions of large vessels in diabetes mellitus is a manifestation of atherosclerosis as it appears also in non diabetics. Only for some reasons the incidence and severity of atherosclerosis should be more pronounced in diabetics—"diabetes enhances the development of atherosclerosis"

There are several reasons today for considering the alternative concept of large vessel disease in combination with atherosclerosis. This consideration implied by the term "diabetic macro-angiopathy emerges from several epidemiological clinical and morphological studies (2, 3 11 14 17 19) The factor(s) responsible for the development of the lesions in the large vessels in diabetes mellitus is not known today

The smooth muscle cell of the arterial media is probably the most important cell type involved in the development of atherosclerosis. More complete consideration of this

subject may be found in recent reviews (8, 9, 25)

It has recently been shown that serum from animals with experimental diabetes mellitus contains a factor(s) which stimulates the arterial medial cells to excessive growth in tissue cultures (14)

The present study was undertaken in order to investigate the growth pattern of rabbit aortic smooth muscle cells grown in *human* serum from young juvenile diabetics and non-diabetics.

The experimental conditions were similar to those used in our study of the effect of rabbit serum on these cells (14)



MATERIAL AND METHODS

Single round primary explants of medial smooth muscle cells were obtained from rabbit aortas by a technique described by *Fischer Dzoga et al.* (5) Thoracic aortas were collected from five normal white male Danish Country rabbits (weight 2–3 kg) Adherent tissue, the adventitia and the intima were removed and explants, 2 mm in diameter were punched out from the remaining media with a skin punch. All manipulations of the tissue were conducted under sterile conditions at room temperature. A total of 60–90 explants could be made from one aorta. Each explants was incubated in 2.5 ml growth medium in a 30 ml plastic tissue culture flask (Falcon) The cultures were grown in filtered sterile atmosphere of 5 per cent CO and 95 per cent air at 37° C The growth medium consisted of a combination of Basal Medium of Eagle (BME) Hank's balanced salt solution, 50 µg/ml of neomycin sulfate and 10 per cent rabbit serum. The medium was changed twice a week Initial growth appeared in about 90 per cent of the explants after 4–8 days of incubation. Cultures not *showing this growth pattern were discarded.*

Experimental Design

The growth of the aortic smooth muscle cell cultures was studied in two experimental situations. In one series of experiments (serum experiments) the growth effect of human serum from diabetics was compared to that of normal serum. In another series of experiments (dialysed serum experiments) the effect on growth of dialysed human diabetic and non-diabetic serum was appraised.

All the experiments were performed after the cultures had developed to the so-called stationary phase in which tissue cultures generally grew much more slowly than the so-called "initial phase This was reached after five weeks of incubation in 10 per cent normal rabbit serum (Fig 1) At this point of time the flasks were

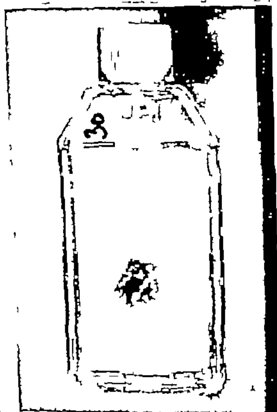


Fig 1 An aortic smooth muscle cell culture in the stationary growth phase i e after five weeks of growth.

TABLE 1 *Serum Experiments Diabetic Subjects*

No.	Age years	Duration years	Glucose mg/100 ml	Cholesterol mg/100 ml	Triglycerides mg/100 ml	Insulin unit/ 24 hours	Retinopathy	Percentage of ideal bodyweight (%)
1	23	17	156	197	71	48 NPH	+	111
2.	25	12	170	129	52	44 NPH	+	106
3	25	12	90	151	68	52 NPH	+	104
4	25	6	227	178	54	52 NPH	—	92
5.	27	1 week	159	242	67	48 NPH	—	99
6.	28	4	208	188	109	50 NPH 12 regular	—	97
7	28	4	130	204	47	40 NPH	—	107
8.	32	24	146	239	74	68 NPH	+	102
9	34	6 month	104	250	105	16 NPH	—	98
10	42	23	221	186	99	30 NPH 8 regular	—	88
Mean	28.9	10.3	161 1	196 4	74.6			100.4
SEM:	2.8	2.8	14.8	12.5	7 1			2.2

The serum values are obtained from the samples used in the experiments

divided into experimental and control groups, care being taken to match each experimental culture with a control of the same size. There were five cultures in each serum group in the serum experiments and ten cultures in each serum group in the dialysed serum experiments. In the experiments with non-dialysed serum three aortas were used but only cultures from the same aorta were paired The growth of the cultures was then continued during the stationary growth phase in

1 five per cent diabetic human serum (non-dialysed dialysed) + 5 per cent normal rabbit serum.

2 five per cent normal human serum (non-dialysed dialysed) + 5 per cent normal rabbit serum.

Sera for the Growth Media

Human diabetic serum was collected from young subjects all of whom were suffering from classical juvenile diabetes mellitus (Table 1) In the serum experiments the sera were provided from ten males with an average age of about 29 years The duration of diabetes varied from 1 week to 24 years. All the diabetics were non-obese and insulin treated. Retinopathy was present among four of the diabetic subjects. In the dialysed serum experiments two diabetic serum pools were obtained from three males and three females, respectively all of whom were young, non-obese and insulin treated (Table 3)

Serum from seven non-diabetic, healthy young, non-obese males was obtained as a control group in the serum experiments (Table 2) In the dialysed serum experiments two pools of normal serum were culled from three males and three females (Table 3) The normal, control subjects were all comparable to the diabetic groups with respect to sex, age and body weight.

Fifty milliliter venous blood was withdrawn from the human subjects after overnight fasting. Insulin (NPH 50) was injected 12 hours before blood sampling in nine of the diabetics and 24 hours before in one. The whole blood was allowed to clot at room temperature for 3 6 hours and the serum was pipeted off after centrifugation at 3000 rpm for 15 min and stored at 25 C. All the manipulations with the serum were conducted under sterile conditions. In case of a defect in the sterile manipulations the serum was sterilized by a membrane filter with a pore diameter of 0.24 µ (Sartorius, W Germany) In these cases the corresponding experimental or control serum was also filtered.

Normal rabbit serum was obtained commercially (Grand Island Biological Co., Scotland)

Dialysis

Pools of diabetic and of non-diabetic serum were dialysed in amounts of 10 ml against 1 liter of Hank's solution changed three times at intervals of twelve hours. The dialysis was succeded by a sterile filtration of the serum through a membrane filter with a pore diameter of 0 24 µ (Sartorius, W Germany)

TABLE 2. *Serum Experiments, Non-diabetic Subjects*

No.	Age years	Glucose mg/100 ml	Cholesterol mg/100 ml	Triglycerides mg/100 ml	Percentage of ideal bodyweight
1	25	90	187	43	95
2.	28	101	180	44	94
	31	80	232	43	93
4	31	91	184	51	100
5	34	90	166	71	107
6.	35	91	188	75	104
7	37	87	167	68	103
Mean	31.6	90.0	186.3	56.4	99.4
SEM	1.6	2.4	8.3	5.4	2.1

The serum values are obtained from the samples used in the experiments.

TABLE 3. *Dialysed Serum Experiments*

	Sex	Number pt.	Age years	Glucose mg/100 ml	Cholesterol mg/100 ml	Triglycerides mg/100 ml
Diabetic subject						
Pool of serum	M	5	32	174	207	81
Pool of serum	F	5.	31	120	197	54
Non-diabetic subjects						
Pool of serum	M	5	32	92	179	59
Pool of serum	F	5	31	65	207	79

The serum values are obtained from the samples used in the experiments

Chemical Methods

Glucose, cholesterol and triglycerides were estimated in an AutoAnalyzer.

Measurements

The *growth rate* of the cultures was followed by determination of their area at days zero, two, four and six after changing to the experimental media. The area was estimated by a point counting technique. A square lattice with squares of 16 mm was placed on the tissue culture flask directly over the growing cultures. The number of cross-points in the grid falling upon the cultures was counted with the grid placed at five different positions. The average number of points was then obtained and utilized as an expression of the area. The increase in the individual culture area was expressed as percentage of the culture area at day zero. The mean per cent increase in the area of each group of cultures was then calculated.

In the experiments with undialysed serum the use of three different sortas resulted in three different growth rates in the cultures. In order to compare the results it was therefore necessary to refer the data to a common baseline for the normal sera, in the following manner: the mean growth rates of the control cultures from the three sortas were termed A_1, A_2 and A_3, respectively. The growth rates obtained from sortas 2 and 3 were referred to that of sorta 1 by multiplying their growth rates in diabetic as well as control sera by factors of A_1/A_2 and A_1/A_3, respectively.

Fig. 4 shows the adjusted mean values of the effect of the individual sera—10 from diabetics and 7 from control subjects—on day 2, 4 and 6 of the experiment.

Autoradiography

In the serum experiments the cell proliferation was estimated from the percentage of cells that had incorporated ^{3}H-thymidine as identified by autoradiography. Four days after the exposure of the cultures to the various growth media 0.1 µCi ^{3}H-thymidine (Amersham, London) per milliliter growth medium were added and the incubation continued for two more days. The cultures were then fixed in formalin. The bottom of each plastic flask was cut out and the formalin fixed cultures

rinsed in water. The slides were dipped briefly in nuclear track emulsion K2 (Ilford) at 24 C, allowed to dry in a horizontal position at room temperature over a period of 3 hours and then transferred to a light tight box and stored in a refrigator for one week. The autoradiograms were developed with D19 (Kodak) at 20° C for 5 min and the slides subsequently rinsed in water and fixed in 30 per cent sodium thiosulfate for 3 min. They were then rinsed carefully in tapwater and stained with haematoxylin for 20 min (Fig. 2). The countings were performed on the peripheral monolayer of the cultures at a total magnification of 00 × Labelled and non-labelled cells were counted in the frame of an ocular grid. A total of 100–300 nuclei were counted from each culture.

In other cultures the percentage of cells in mitosis after the completion of the experimental phase was determinated histologically as follows. The growth media were removed and the cultures washed in phosphate-buffer. The bottom of the flasks were cut out and handled as regular histological preparations. The cells were stained with haematoxylin for 20 minutes and the number of mitotic and non-mitotic cells counted blindly within the same grid as used before. The grid was applied 24 times around the peripheral growth zone of each culture and included a total of 400–700 cells per culture.

Statistical Methods

The statistical comparison—paired and non-paired—was carried out by means of the Student's t-test. Ratios between labelled and non-labelled cells as well as between mitotic and non-mitotic cells were evaluated statistically with a χ^2-test. A 2p-values less than 0.05 was accepted as the limit of significance.



Fig. 2. An aortic smooth muscle cell culture labelled with ^{3}H-thymidine and identified by autoradiography.

RESULTS

The concentration of cholesterol, triglycerides and glucose in the diabetic and non-diabetic serum is shown in Table 1 and 2. The glucose concentration was on a statistical basis significantly higher in the diabetic than in the non-diabetic serum ($2p < 0.01$). The concentration of the lipids in the diabetic serum was about the same as in the non-diabetic serum.

Serum Experiments

The outgrowth of smooth muscle cells from the primary explants propagated in human diabetic serum was significantly larger than in the control group ($2p < 0.01$, Fig. 3) and this difference was already evident after two days of incubation. Furthermore no differences were found between sera from patients with long or short term diabetes.

There was no correspondence between the concentration of glucose and the outgrowth of smooth muscle cells in diabetic serum or between the amount of injected insulin and the growth of the cultures.

Dialysed Serum Experiments

The growth of aortic smooth muscle cells measured at day 3, 5 and 7 was appreciably increased in the presence of dialysed diabetic serum as compared to that in dialysed non-

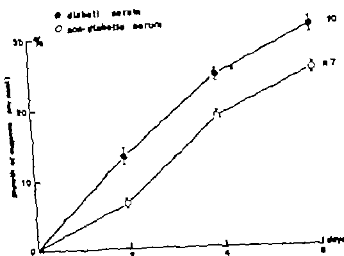


Fig. 3 The percentage growth of rabbit aortic smooth muscle cell cultures grown in human diabetic and non-diabetic sera in the stationary phase of growth. The bars represents 1 S.E.M.

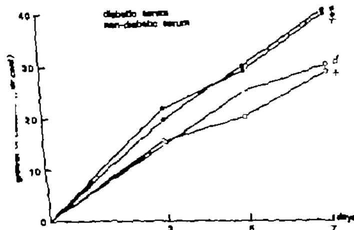


Fig. 4 The percentage growth of rabbit aortic smooth muscle cell cultures incubated in dialysed pools of human diabetic and non-diabetic sera in the stationary growth phase. The pools were obtained from three men and three women, respectively.

TABLE 4

	Non-diabetic serum	Diabetic serum	
Autoradiography			
Number of cultures	5	10	
Total number of cells counted	1410	377*	
Percentage of labelled cells	53.9 %	62.2 %	$2p<0.005$
Mitosis studies			
Number of cultures	5	7	
Total number of cells counted	2651	3149	
Percentage of cells in mitosis	12.2 %	17.9 %	$2p<0.005$

diabetic serum ($2p<0.05$ Fig. 4). This phenomenon held true for serum from both males and females.

Cell proliferation

In accordance with the observations on growth rates the number of cells labelled with ³H thymidine was significantly higher

after incubation in human diabetic serum than in cultures incubated in control serum and so also was the percentage of cells in mitosis ($2p<0.005$, Table 4).

DISCUSSION

In an earlier study an accentuation of the growth of aortic smooth muscle cells was shown to occur in serum from rabbits with experimental diabetes mellitus, in the initial as well as in the stationary phase of growth (14). The results reported here, performed during the stationary growth phase demonstrated that the same acceleration of growth occurs when *human* serum from diabetic patients is used in the incubation medium.

The cell cultures in the present report consist of aortic smooth muscle cells grown out from primary explants from rabbit's aorta. The isolation technique has been the same as used by *Fischer Dzoga et al.* (5) whose observations based on employing immunohistochemistry and electronmicroscopy indicated that the outgrowing cells are smooth muscle cells. In the present study electronmicroscopy performed occasionally on the cultures has been in accordance with the observations of *Fischer Dzoga et al.* (5).

There was no correlation between the growth rate and the glucose concentration in the diabetic sera. However the diabetic state was almost of the same degree in the individual serum-donors and fairly well controlled. Whether there is any difference between the growth effect of serum from very strictly controlled and from very badly controlled diabetics remains to be established.

The factor or factors which enhanced the growth was present in the serum of patients with varying durations of diabetes and present—even after only 1 week of affliction.

In an *in vitro* study *Morrison et al.* (18) found a decrease in oxygen consumption and an increased level of fructose in intima media preparations of rabbit aortas incubated in media containing increasing amounts of glucose. *Arnqvist* (1) was also able to demonstrate an increased elaboration of lactate of intima media preparations of arteries incubated in media with high and low levels of glucose. It is indicated from these two studies that glucose concentration has a direct effect on the glucose metabolism in the vessel wall. This conclusion is not in consistent, however with the present findings which show that the removal of glucose from diabetic sera by dialysis did not influence their growth stimulating capacity. This result is in accordance with those obtained from the experiments in which glucose was added to non-diabetic rabbit serum. In these studies no enhancement of growth was observed (14).

The experiments performed with dialysed serum also reveal that the growth promoting factor(s) in the diabetic serum are not amino-acids, fructose or ketones since these substances will be removed from the serum during the dialysis. As the factor(s) remain in the dialysis-bag the molecular weight must be higher than 3000–4000.

The diabetic serum was collected from subjects who had all been on insulin treatment from a few weeks to several years. It has been proposed that insulin is involved in the development of large vessel abnormalities in diabetes mellitus and atherosclerosis (23, 24). However there was no relationship between the growth promoting effect of the sera and the amount of injected insulin in the diabetics. Moreover normal human serum supplemented with 50–2000 µU insulin per milliliter did not enhance growth of the aortic myomedial cells (13).

It has previously been shown that the growth of arterial medial cells can be accelerated by addition of hyperlipemic serum to the growth medium (4, 6, 10, 14). As there was virtually no difference in the serum concentration of cholesterol and triglycerides between the sera from the diabetics and controls the observed stimulation of growth cannot be imputed to the amount of lipid. However the effect on growth of apoprotein isolated from human diabetic lipoproteins has yet to be elucidated.

Recently it has been reported that platelets

from non-diabetic human serum contain a factor(s) which stimulated excessive growth of aortic smooth muscle cells from monkeys (21), although in this case trypsin treated cells were used for the investigation. A study of the effect of diabetic serum before and after removal of thrombocytes, using non trypsinized primary cell cultures is under way in this laboratory.

The results obtained by autoradiography and counts of mitotic cells suggest that diabetic serum increases cell proliferation. Similar results were obtained after growth in diabetic *rabbit* serum (14). In an *in vivo* study of the growth of kidney cells, using autoradiography as well as mitosis count *Pederson* & *Gelfand* (20) found a discrepancy between the results obtained with these two techniques. They concluded that some cells, after synthesizing DNA are arrested in an intermediary phase (G2) before going into mitosis. In the present study the increase in growth of the cultures of ^3H thymidine and in mitotic frequency occurred simultaneously in cells grown in media containing diabetic serum. Assuming that the length of the G2-phase and the mitosis phase is unchanged, the present results suggests that diabetic human serum induces true cell-proliferation.

The causes for the development of diabetic angiopathy are still obscure. However it has been suggested that growth hormone is a causal factor for the development of vessel disease in diabetes mellitus (16). It is, of course, hazardous to extend the obtained results to the diabetic macro- and micro-angiopathy in humans. However, it is noteworthy that the number of cells in tunica media in the small intracardial branches of the coronary arteries is significantly increased in patients with juvenile diabetes mellitus (12). Moreover in a recent study addition of human growth hormone to normal human serum promoted growth of the aortic medial cell cultures. The growth effect was obtained with an ambient growth hormone concentration of 1 ng per milliliter medium (13).

At the moment further experiments aimed at the characterization of the growth factor(s) in the human diabetic serum are in progress in this laboratory.

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INTRACELLULAR CYSTS IN GASTRIC CARCINOMA

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Nevalainen, T J & Järvi, O H. Intracellular cysts in gastric carcinoma. Acta path. microbiol. scand. Sect. A, 84 517-522 1976.

Intracellular cysts were often found in the tumour cells in gastric carcinomas of intestinal as well as diffuse type in specimens from stomachs resected for gastric cancer In the light microscope, the cysts appeared usually as solitary cytoplasmic vacuoles. In the electron microscope the intracellular cysts were seen as round cavities, often containing homogenous or granular mucous material which stained with the periodic acid-silver methenamine (PASM) method Relatively long microvilli were lining the cysts and, not infrequently abundant cytoplasmic microfilaments would surround the cyst wall. Similar cysts have been observed in a number of other tumours and, accordingly their diagnostic significance must be considered in connection with other methods such as differential staining of cellular mucosubstances.

Key words Electron microscopy gastric cancer microvilli mucosubstances

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It has recently been described that intracellular cysts occur in breast carcinoma cells (1) and in tumour cells in the pleural or peritoneal fluid in patients with breast carcinoma (20) Their presence was considered to be helpful in the diagnosis of breast carcinoma, especially if identified in large numbers when they might provide additional support for the breast origin of a metastatic tumour In our studies on the fine structure of gastric carcinoma (7 8, 14) we have observed similar intracellular cysts in carcinomas of intestinal and diffuse type. In addition, a review of the literature discloses that these structures cannot be considered as unique features of breast or gastric carcinoma cells since they are found also in a number of other tumours. The ultrastructure of the intracellular cysts in gastric carcinoma cells is described in the present communication.

MATERIAL AND METHODS

Specimens from 47 stomachs resected for gastric carcinoma were studied by light and electron microscopy Samples from each stomach to be histologically examined were processed according to the routine by formalin fixation and paraffin embedding. Sections were stained by H E, van Gieson, Alcian blue pH 2.5-PAS and High Iron Diamine Alcian blue (17) techniques.

Samples for electron microscopy were fixed in 3 per cent glutaraldehyde in 0.1 M cacodylate buffer at pH 7 4 (19) for 3-24 hours and postfixed in 1 per cent osmium tetroxide (12) for 1 hr The tissues were washed in the buffer dehydrated in ethanol, and embedded in Epon 812 (11) Thin sections were stained with uranyl acetate (24) and

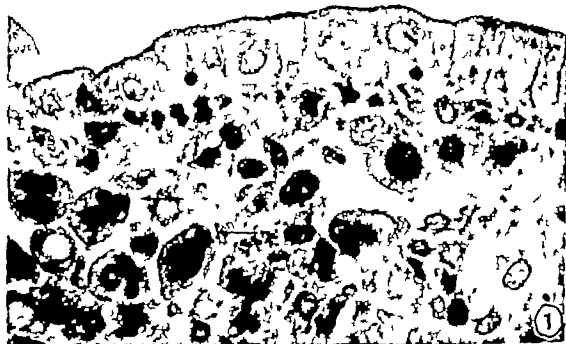


Fig 1 Light micrograph of a diffuse gastric carcinoma containing numerous tumour cells with intracellular cysts (arrows) Toluidine blue-stained, 1 μm thick Epon section. Mag. 650 ×

lead citrate (22) and studied in a JEOL JEM 100C electron microscope. For purposes of orientation, semithin (1 μm) Epon sections for light microscopy were stained with alkaline toluidine blue or PAS (15 16) Mucosubstances were demonstrated in the electron microscope by the periodic acid-silver methenamine (PASM) technique (15)

RESULTS

In the light microscopical preparations the intracellular cysts appeared as solitary large cytoplasmic vacuoles (Fig 1) Sometimes they occurred in the majority of the cells in a given tumour

In the electron microscope, the intracellular cysts were seen as round cytoplasmic cavities which did not communicate with the cell exterior (Figs. 2 and 3) The nucleus was located at one side of the cell and the usual cell organelles such as mitochondria and profiles of the endoplasmic reticulum were present in the cytoplasm. Numerous cytoplasmic microfilaments were often surrounding the cysts with relatively long microvilli lining the cyst wall. The microvilli contained a more dense microfilamentous core (Fig 3) The cysts contained homogenous and granular material occasionally with membranous components (Figs 2 and 3) The cyst contents reacted strongly in the PASM-stained sections (Fig 4)

DISCUSSION

Intracellular cysts with brush border and secretion in gastric carcinoma cells were first described by *Järvi & Laurén* in 1951 (7) who had observed such cysts in the intestinal as well as the diffuse type of gastric carcinoma. The first electron microscopical findings concerning these cysts were those reported by *Cusek & Krause* (5) while *Kondo et al* (9) described the phenomenon in greater detail. The findings obtained in earlier studies were largely confirmed in the present ultrastructural study The cysts were solitary vacuoles without any communication with the exterior of the cell and they were found both in intestinal and diffuse types of gastric carcinomas. The cysts contained



Fig. 2. Intracellular cysts lined by relatively tall microvilli and containing granular and flocculent material. Stained with uranyl acetate and lead citrate. Mag. 9,500 ×

homogenous and finely granular material which was mucinous in nature since it stained with the PASM-method. The most characteristic feature of these cysts was the well-developed microvillar lining on the cyst wall. This ultrastructural feature clearly differentiates these cysts from the mucous vacuoles seen in the signet ring cells of some gastric carcinomas of diffuse type (14).

The histogenetic role of the intracellular cysts in the gastric carcinoma cells remains unknown. It has been postulated by *Järvi et al.* (8) that the cells with microcysts containing mucus should correspond to poorly developed goblet cells the differentiation of which in some way had been distorted. This assumption, however, is contradicted by the occurrence of the microvillous brush border on the cyst wall. It is rather to be considered that these gastric carcinoma cells are expressions of a lost surface polarity of the cells, in other words, that the cells are building surface structures inside the cytoplasm. The accumulation of secretion products around and in the cysts is a logical consequence in cells where the capacity of secretion is still preserved.

As regards the fine structure, the microvilli lining the cyst wall were similar to the microvilli found in abundance on the plasma mem-



Fig 3 The intracellular cyst contains granular material and a dense body. Microfilaments are surrounding the cyst wall and microvilli are projecting into the cyst lumen. Stained with uranyl acetate and lead citrate. Mag. 12,000 ×

brane of intestinal type and of some specimens of gastric carcinoma cells of diffuse type (14). Furthermore similar microvilli are typical of the columnar epithelial cells in the intestinalized stomach epithelium. Thus the presence of microvilli on the cyst wall of carcinoma cells implies a histogenetic role of intestinal metaplasia in the development of gastric carcinoma (8).

The intracellular cysts have been considered to be characteristic structures of gastric carcinoma cells (7, 8, 9). They are common also in carcinomas of the gut (6). *Laurén* however interpreted the cysts as poorly developed goblet formations (10). Very similar inclusions were found in the breast carcinoma tissue by *Battifora* (1) and in the tumour cells in the pleural and peritoneal fluid in patients with breast carcinoma by *Spriggs* & *Jerrome* (20) who proposed that their identification might be a means by which to provide support for the breast origin of a metastatic tumour. However the diagnostic significance of these cysts as such is questionable, since very similar structures have been observed in a wide variety of tumour cells, including granular cell adenocarcinoma of the kidney (18), mesothelioma (23), adenomatoid tumour (21) and cultivated JB-1 ascites tumour cells (2, 3, 4). However in connection with other methods, e.g. differential staining of mucosubstances, the microcysts could be of diagnostic value. In addition, a more detailed study of the microvillar structures could contribute to a more specific interpretation of the cysts.

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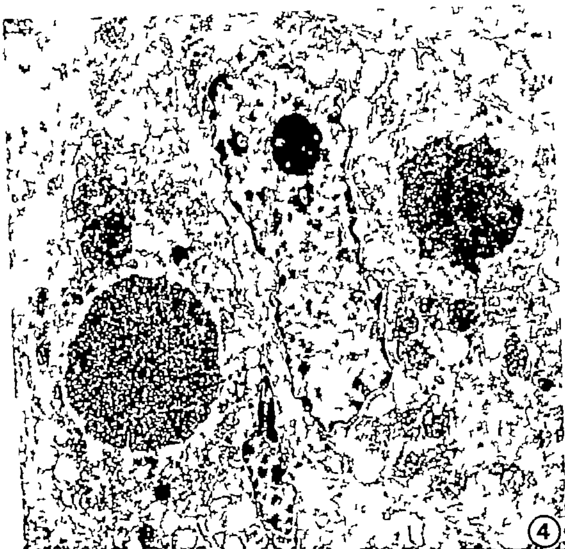


Fig. 4 Intracellular cysts containing mucous material. PASM-stained section. Mag. 7,000 ×

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EVIDENCE FOR AN INITIAL, THYMUS INDEPENDENT AND A CHRONIC, THYMUS DEPENDENT PHASE OF DOCA AND SALT HYPERTENSION IN MICE

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Svendsen, U. G. Evidence for an initial, thymus independent and a chronic, thymus dependent phase of DOCA and salt hypertension in mice. Acta path. microbiol. scand. Sect. A, 84 523–528, 1976.

Treatment with desoxycorticosterone acetate (DOCA) and 1 per cent saline as drinking water for 21 days caused a significant and similar increase in blood pressure in haired mice, with a normal thymus function, as in nude mice with genetical aplasia of the thymus. After 57 and 78 days there was, however, a significantly more pronounced increase in blood pressure in haired than in nude mice. A marked degree of round cell infiltration around intrarenal vessels and degenerative changes including wedge-shaped infarcts were observed in the kidneys of the haired mice, commencing after 57 days of treatment, while no such changes were found in nude mice. Thymus grafting in nude mice, successively treated with DOCA and salt, conferred the ability to react with chronic hypertension and intrarenal vascular disease, equal to the reaction seen in haired mice. The present investigation has provided evidence for the existence of an initial thymus independent and chronic thymus dependent phase of DOCA and salt hypertension in mice. It still remains an unsolved problem whether the secondary blood pressure fall observed in nude athymic mice is a direct consequence of the lack of perivascular cellular immune reactions, or caused by other defects in this strain of mice.

Key words: Hypertension, salt, DOCA, thymus, mice.

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A perivascular round cell infiltration around hypertensively damaged arteries form a characteristic part of the hypertensive vascular disease both in human beings (for literature see 5) and in experimental animals (4). The morphology of the infiltrates are similar to the morphology of the cellular reactions in immune reactions of the delayed type for which thymus and the thymus-derived lymphocytes (T-cells) are necessary (6, 14). The existence of thymus dependency of the round cell infiltrations has been demonstrated in previous studies using different models of acute and chronic experimental hypertension (4, 9, 11, 12). Experiments with "Loomis" hypertensive mice (one kidney partly infarcted by ligation of the anterior branch of the renal artery and contralateral nephrectomy (3)) have provided some evidence that

the presence of thymus may be essential not only for the cellular reaction but also for the late phase of hypertension athymic nude mice (see 7) failed to maintain the high blood pressure for more than 90 days, in contrast to normal mice in which a marked degree of hypertension and vascular disease occurred even after 120 days (12)

The aim of the present study was to investigate whether or not a similar chronic thymus dependent phase of hypertension could also be demonstrated in DOCA/salt hypertensive mice. For this purpose normal, athymic nude and thymus transplanted nude mice were treated with DOCA and salt. Randomly selected groups were investigated after 21 57 and 78 days.

MATERIAL AND METHODS

Animals outbred NMRI mice (SPF GL Bomholt gård Ltd., Ry Denmark) 20–25 g, 6–7 weeks of age, carrying the mutant allele (nu/nu) and their haired littermates (nu/+) received tetracyclin (Tetracyclin Novo Vet., 100 mg/l) in the drinking water About equal numbers of males and females were used, the sex not playing any role for the results.

Thymus transplantation subcutaneous whole thymus grafts were made in 6-week-old nude mice. Donors were NMRI mice, less than 24 hours old. The success of the thymus-transplantation was judged from the achieved ability to reject a skin transplant (Donors C_3H/Tif/Bom. mice)

Skin transplantation was performed as previously described (12) Ten nude and all thymus transplanted nude mice (4 weeks after the transplantation) were challenged. The animals were grouped according to the treatment

Group 1 (controls) 20 nude and 20 haired untouched control mice 3–6 months of age. In addition, 8 nude mice, 6–7 months of age, which had been thymus transplanted and successively challenged with and had rejected an allograft. In 10 nude and 10 haired mice, the daily tapwater intake was determined for 14 days, as described below

Group 2 (DOCA and salt treated) 41 haired, 41 nude and 18 female thymus transplanted nude mice, which had been able to reject a skin allograft, were unilaterally nephrectomized. One week later they received 1 per cent saline as drinking water and 6 mg DOCA (desoxycorticosterone acetate Percorten® microcrystalline CIBA) was applied subcutaneously once a week. Randomly selected groups of mice were investigated after 21 57 and 78 days of treatment. Thymus transplanted nude mice were only investigated after 57 and 78 days. The daily saline intake of the individual mice, one in each cage, was determined 14–21 days before the blood pressure was recorded by measuring the disappearance of saline from the drinking bottles.

The further treatment was the same in groups 1 and 2. Before sacrifice, the blood pressure was recorded. In light ether anaesthesia, a catheter was placed in the left carotid artery and connected to a Tyberg Hansen capacitance pressure transducer (Simonsen & Weel, Copenhagen) and a G-14 graphic recorder (Model G-14 Danbridge, Copenhagen) the blood pressure was subsequently recorded for one hour in the conscious semirestrained animal while it was placed in a transparent plastic tube. After termination of the blood pressure recordings, *arterial blood samples* were obtained for determination of the haematocrit and the number of circulating lymphocytes. After sacrifice, the relative heart weight and the kidney weight were determined. The heart, kidney pancreas, the small intestine, and, in the case of the thymus transplanted nude mice, also the subcutaneous thymus graft, were fixed in 4 per cent formaline and embedded in paraffin. Arterioles situated in the mesenterium and the submucosa were isolated from the small intestine of 5 haired mice treated for 78 days Five micron-thick sections (serial sections from mesenteral arterioles) were cut and stained with van Gieson Hansen (VGH) and the periodic acid Schiff (PAS) stain. The round cell infiltrations around arteries in the heart and both the interlobar and the interlobular/arcuate arteries of the kidney were graded semiquantitatively according to photographed scales from 1+ to 3+ in which a 1+ cellular reaction includes the sparse mononuclear reaction in adventitia of normal arteries (10, 11)

For comparison of experimental results, the student's t-test was used. The five per cent level was used as indicative for significance of differences.

RESULTS

A subcutaneous macroscopically visible thymus was observed in all the thymus transplanted nude mice which rejected the skin allografts within an average of 20 days (range 14–35 days) None of the 10 nude mice without a thymus graft were able to reject the skin allograft, and donor hair grew on the graft by the time when the experiment was terminated (78 days)

Group 1 control mice Fig 1 shows the *mean blood pressure* in the 40 nude and

haired mice (range 90–140 mm Hg). Although the mean blood pressure was the same ($p>0.5$) the mean *relative heart weight* in the nude mice was 0.43 ± 0.01 (SEM) per cent (range 0.35–0.54) significantly greater ($p<0.005$) than in the haired mice (0.35 ± 0.01 per cent (range 0.33–0.43)). Thymus transplanted nude mice had the same mean blood pressure (119 ± 6 mm Hg (range 90–140) $p>0.3$) and the same mean relative heart weight (0.39 ± 0.04 per cent (range 0.33–0.43) $p>0.05$) as the nude control mice. The mean number of *circulating lymphocytes* was 2325 ± 279 per microlitre (range 1400–4425) in the nude, and 5295 ± 323 per microlitre in the haired mice (range 4400–7525) significantly ($p<0.001$) greater than in the nude mice. In thymus transplanted nude mice it was found to be 2720 ± 634 per microlitre (range 1450–6450) similar ($p>0.5$) to the value obtained in the nude mice. The *haemotocrit* ranged around 40–45 per cent in all animals. The daily intake of tap-water was between 6 and 10 ml.

Microscopic investigation: the heart and pancreas: no vascular disease. In *the kidney* no vascular disease, except that 4 of the 8 thymus transplanted nude mice which had had the thymus for 5–6 months presented a slight increase (less than a 2+ reaction) in round cell infiltrations around the afferent arterioles and the interlobular arteries; it was of the same morphology but much less intense than that found in hypertensive mice. No other degenerative changes were found.

Group 2. DOCA and salt-treated mice: 8 nude, 7 haired and 2 thymus transplanted mice died spontaneously after commencement of the treatment. All of the surviving mice were without clinical symptoms of disease. Fig. 1 shows *the mean blood pressure* in the DOCA and salt treated mice compared with the mean blood pressure obtained in the nude and haired control mice. After 21 days a significant increase in mean blood pressure was observed, being similar in nude and haired mice (range 125–180 and 130–170 mm Hg respectively). On day 57 a significant difference between nude and haired

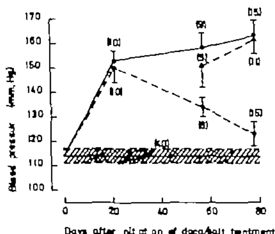


Fig. 1. The mean blood pressure $\pm$ SEM in haired (●——●) nude (○- - -○) and nude thymus transplanted (▲- - -▲) mice treated with DOCA and salt. The scratched area indicates the mean blood pressure in nude and haired control mice $\pm$ SEM. A significant ($p<0.001$) increase in blood pressure was observed in all groups of DOCA/salt treated mice, except in nude mice after 78 days of treatment ($p \approx 0.05$). After 78 days of treatment a significant ($p<0.001$) difference between haired and nude thymus transplanted DOCA and salt treated mice, as compared with the similarly treated nude mice was observed. Number in brackets number of mice.

mice was found (range 125–150 and 130–185 mm Hg, respectively) in spite of the fact that the mean arterial pressure in both groups was significantly increased as compared with that in the control mice. On day 78 the mean blood pressure in animals in the group of nude mice was significantly lower than that in the DOCA and salt treated haired mice (range 100–150 and 140–210 mm Hg respectively) and had decreased to a value which differed from that of the controls to a degree only bordering on significance. In thymus transplanted nude mice, the mean blood pressure after 57 and 78 days (range 120–170 and 140–185 mm Hg, respectively) of treatment was similar ($p>0.5$) to that in the haired DOCA and salt treated groups of mice. The *mean relative heart weight* was significantly increased both in nude (0.50 ± 0.01 per cent (range 0.44–0.56) $p<0.005$) and in haired (0.45 ± 0.01 per cent (range 0.41–0.53) $p<0.001$) mice already after 21

TABLE 1 *Survey of the Experimental Results*

Days	a. interlobaris			a. interlobularis/arcuata		
	Haired	Nude	Nude + TH	*Haired*	Nude	Nude + TH
	+	+		+	+	
	+	+		+ +	+	
	+	+		+ +	+	
	+	+		+	+	
	+	+		+ +	+	
21		+		+	+	
	+	+		+	+	
	+	+		+	+	
	+	+		+	+	
	+	+		+	+	
		+	+	+	+	+
	+	+	+	+ +	+	+ +
	+	+	+ +	+	+	+ + +
	+	+	+	+	+	+ +
3	+	+	+	+	+	+ +
	+	+		+ +	+	
	+	+		+	+	
	+	+		+ + +	+	
	+			+ +		
	+	+	+ + +	+ + +	+	+ + +
	+ +	+	+	+	+	+
	+	+	+	+ +	+	+ +
	+	+	+ + +	+ +	+	+ + +
		+	+ + +	+ +	+	+ + +
	+	+	+	+ + +	+	+ +
	+ + +	+	+ +	+	+	+ +
	+ + +	+	+	+ +	+	+
78	+	+	+	+ +	+	+ + +
	+	+	+ + +	+ +	+	+ + +
	+ +	+	+	+ +	+	+ +
	+	+		+ +	+	
	+ + +	+		+ + +	+	
	+ + +	+		+ + +	+	
	+	+		+ + +	+	

Table 1 shows the degree of round cell infiltration around the interlobar and the interlobular/arcuate arteries in the mice treated with DOCA and salt for 21 37 and 78 days. A marked difference between the cellular reaction in haired and nude mice is found after 78 days of treatment. Cellular reactions in nude thymus transplanted mice are similar to those in haired mice after 37 and 78 days of treatment.

days. *The daily saline intake* increased similarly (mean 35 ml/day/25 g mouse after 21 days of treatment) in nude and haired mice during the first 3 weeks of treatment. Thereafter the nude mice maintained this increased level unchanged while an even higher level of saline intake was observed in the haired and the thymus transplanted nude mice after 57 and 78 days of treatment (mean 60 ml/day/25 g mouse). *The kidneys* were found enlarged and were of a pale grey colour. While the surface of kidneys in the nude mice was always smooth, a granulated surface was often found in haired and thymus transplanted nude mice.

Microscopic investigations. *The kidney*

Table 1 shows the degree of round cell infiltration around the interlobar and the arcuate/interlobular arteries. The infiltrates consisted mostly of cells the morphology of which was like that of lymphocytes and monocytes, but granulocytes and plasma cells were also present. Increased numbers of round cells were found both in haired and nude thymus transplanted mice after 57 and (most severe) after 78 days of treatment, but not in the nude mice. In haired and nude thymus transplanted mice, increased numbers of round cells were frequently found around the afferent arterioles after 57 and 78 days of treatment. Increased amounts of connective tissue fibrils lying around the arteries, increased amounts of PAS positive material in the glomerular tufts, dilatations and degenerations of tubules with hyaline casts and wedge-shaped infarcts were found after 78 days. All mice which died spontaneously had marked (2+ or more) round cell infiltrates around the intrarenal vessels. Only few lesions, mostly appearing as moderately increased amounts of PAS positive material in the glomerular tufts, were found in nude mice, both in mice which were sacrificed and in mice which died spontaneously. *The heart*: vascular lesions consisting of hypertrophia of the media, fibrinoid degeneration and focal myocardial necroses were observed in 2 haired and 3 nude mice after 21 days, in 4 haired 2 nude thymus transplanted and no nude mice after 57 days, and in 12 haired, 4 nude thymus transplanted and 3 nude mice after 78 days of treatment. Round cell infiltrations around the vessels were observed in 5 haired mice (2+ cellular reactions). *The pancreas*: an increased amount of PAS positive material in the vessel walls and a slight increase in perivascular round cell infiltrations were observed in a few haired and nude thymus transplanted mice, but not in nude mice. *Mesenterial arterioles*: fibrinoid necrosis and increased perivascular round cell infiltrations were observed in arterioles from 2 mice.



Fig. 2. A wedge-shaped infarcted area from the kidney of a nude thymus transplanted mouse treated for 78 days with DOCA and salt. Degenerative changes, consisting of increased PAS positivity of the glomerular tufts, dilated and degenerated tubuli with hyaline casts and increased amounts of interstitial connective tissue, are apparent. The arrow indicates a 3+ cellular infiltration around an interlobular artery. PAS staining. 56 ×

DISCUSSION

The development and level of DOCA and salt induced hypertension in the haired mice used in the present study was similar to that in DOCA and salt hypertensive mice studied by other investigators who utilized a tail cuff method for blood pressure measurements (1). The intrarenal vascular disease in the haired mice was equal to that described previously (10) in mice and rats (2, 8). Contrary to the findings in the haired mice, nude mice were unable to maintain an elevated blood pressure in spite of continued treatment with DOCA and salt. Nude mice also failed to develop

renal vascular disease. Transplantation of thymus into nude mice not only induced the ability to react with delayed type immune reactions, but also induced the ability to maintain a high pressure and to develop typical renal vascular disease. These findings tentatively suggest that the maintenance of elevated systemic arterial pressure in the late phase of DOCA-salt hypertension might be causally related to the secondary thymus-dependent vascular lesions, and due to progressive ischaemia, resulting from multiple constrictions of the small intrarenal arterial vessels. Inflammatory oedema of the media may also contribute to reduce the vessel lumen (cf. 10) As far as the blood pressure is concerned, results similar to those obtained in the present study have been obtained in another strain (C57/bl/6j) of nude and haired mice in which the kidneys were partly infarcted (13) but in this latter strain, only few intrarenal round cell infiltrations were found, suggesting either that the thymus may also be important for cellular (or other?) reactions in other areas of the circulatory system which may be of pathogenic importance for the hypertension or that the cellular reaction is of little, if any significance for the high blood pressure.

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HISTOLOGICAL TYPING OF LUNG CANCER

Application of the World Health Organization Classification to 479 Cases

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Larsson, S & Zettergren, L. Histological typing of lung cancer Application of the World Health Organization classification to 479 cases. Acta path. microbiol. scand. Sect. A, 84 529–537 1976

Four hundred and seventy nine primary lung cancers were typed according to the WHO histological classification. The character of the material and the methods of investigation are described. All patients had been subjected to mediastinoscopy and 313 patients had been operated upon. Nearly half of the tumours (48 per cent) was epidermoid carcinomas. Small cell anaplastic carcinoma occurred in 25 per cent and around two thirds of these were of oat cell type. Adenocarcinoma was found in 22 per cent and the acinar type predominated. Bronchiolo-alveolar carcinoma occurred in 1 per cent and large cell carcinoma in 3 per cent. Typing of biopsy specimens was made in 289 cases in which a positive biopsy had been obtained during the pretreatment period. The result of the biopsy typing was checked against that of the final one. In the total group the preoperative histological diagnosis tallied with the final one in 88 per cent. In patients who had been subjected to surgery the pretreatment diagnosis of epidermoid carcinoma was correct in 86 per cent, that of small cell anaplastic carcinoma in 92 per cent and that of adenocarcinoma in 100 per cent. The consistency was also high in the category of patients not subjected to surgery Despite their critical attitude towards the delimitation of epidermoid carcinoma in he WHO-classification the present authors find it to be a reliable guide to routine typing of lung cancer

Key words Lung cancer; histological typing.

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In the present investigation the WHO histological classification of lung cancer has been applied to a series of such tumours in order to evaluate the practicability of the proposed system. The present investigation also deals with the reliability of biopsy typing of lung cancer according to the WHO classification. A study of the biological characteristics of the histological tumour types in the present series has already been published (*Larsson* 1973)

MATERIAL AND METHODS

The material accessible for classification derives from 479 patients with primary lung cancer In all cases a microscopic examination was carried out on *material obtained from various sites*. Biopsy was performed at bronchoscopy in 274 cases, at mediastinoscopy in 452 cases and at thoracoscopy in 23 cases. Peripheral tumour metastases were extirpated in 7 patients in connection with the investigation and in an additional 33 cases at a later stage in the development of the illness. In 29 cases biopsy was performed at thoracotomy and in 277 cases the extirpated lung tumour was microscopically examined.

TABLE 1 *Methods Used in Obtaining Material for Pretreatment Histological Typing*

Method(s)	Operated patients No.	%	Non operated patients No.	%
Bronchoscopy	97	64.2	26	18.8
Mediastinoscopy	31	20.5	67	48.6
Thoracoscopy	0	0	1	0.7
Biopsy of metastasis	2	1.3	2	1.4
Mediastinoscopy and other methods	21	13.0	42	30.4
Total	151	100.0	138	100.0

All histological material was preserved embedded in paraffin and had been routinely examined by pathologists in the department. The slides were all re-examined. As some of the slides had faded or were otherwise defective they were either re-stained or new slides were made.

The routine staining used was hematoxylin-eosin and/or hematoxylin-v Gieson. In a number of cases additional staining was carried out using mostly PAS and a combined staining of keratin and mucin (haemalum-erythrosin-saffron (Masson) and alcian green).

The histological material from each patient was examined in a pre-determined sequence bronchoscopy material, mediastinoscopy material, other biopsy material, resected tumours and finally autopsy material. Even slides primarily listed as negative with regard to the presence of malignant tumour were re-examined. Typing of the tumours was done blindly i.e. without knowledge of clinical data and previous diagnosis. The degree of differentiation was recorded as high or low.

After classification the histological material was divided into two groups. All biopsies performed prior to treatment were placed in one group ("biopsy group"). In the other group ("final material group") the remainder of the material was recorded. The purpose of this division was to check the reliability of biopsy typing by comparing the result of this typing with that of the final classification of the tumour.

The biopsy group consisted of 355 positive biopsies, of which 182 were performed at bronchoscopy and 161 at mediastinoscopy. The biopsies were from 289 patients. The mediastinal biopsies comprised 151 lymph nodes with cancer growth, 9 primary tumours and one sample of fat tissue with tumour embolies in the lymphatic vessels.

Five biopsies done at thoracoscopy derived from the primary tumour or pleural metastases. In 7 cases the biopsy came from a distant metastasis.

As Table 1 illustrates, in 63 cases (22 per cent) two or more biopsies were positive while in 123 cases (43 per cent) only biopsy at bronchoscopy and in 98 cases (34 per cent) only biopsy at mediastinoscopy resulted in a tumour diagnosis. In the operated patients biopsy was positive in 48 per cent and in the non-operated in 80 per cent. In 64 per cent of the operated patients biopsy at bronchoscopy was positive compared to only 19 per cent of the non-operated. On the other hand biopsy at mediastinoscopy was rated positive in only 20.5 per cent of the operated patients as opposed to 49 per cent of the non-operated.

The final material group consisted of 277 resected tumours, 27 biopsies made at thoracotomy, 53 biopsies made after introduction of therapy and 259 autopsy specimens.

As it was suspected that radiotherapy and/or cytostatic treatment as well as postmortem changes might influence the reliability of classification, the result of pre-operative biopsy typing was compared with that of the tumour classification in 146 operated and 100 non-operated patients.

RESULTS

Histological Type of All Tumours

This classification was based upon examination of all available material from the 479 patients. The result is presented in Table 2.

Fig. 1 Highly differentiated epidermoid carcinoma with production of keratin. Hematoxylin-v.Gieson.

Fig. 2 Small cell anaplastic carcinoma, fusiform cell type. Hematoxylin-eosin.

Fig. 3 Small cell anaplastic carcinoma, polygonal cell type. Hematoxylin-v.Gieson.

Fig. 4 Small cell anaplastic carcinoma, oat cell type. Hematoxylin-eosin.

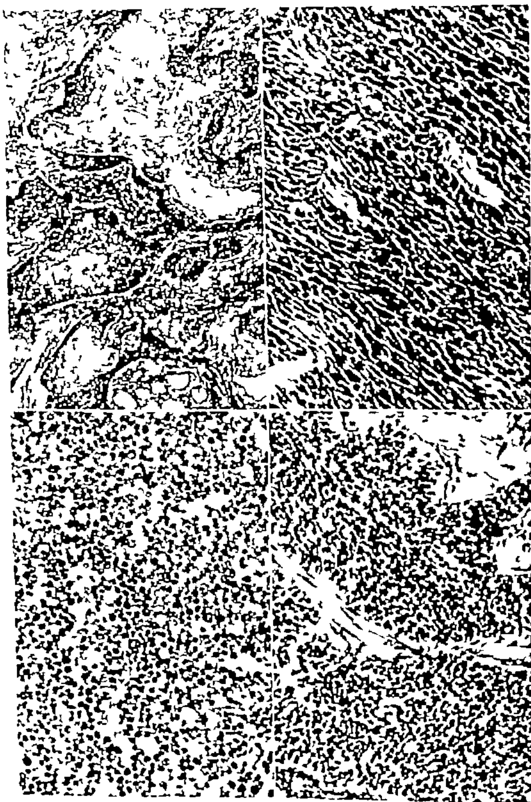


TABLE 2. *Tumour Type Distribution in 479 Cases of Primary Lung Cancer. Percentages Are Based on the Grand Total. Coding of Tumour Categories According to WHO*

Histological type	No.	%
I. Epidermoid carcinoma	231	48.2
II. Small cell anaplastic carcinoma	121	25.3
1 fusiform cell type	*12*	*2.5*
2. polygonal cell type	*35*	*7.3*
3 oat cell type	*71*	*14.8*
4 other type	*3*	*0.6*
III. Adenocarcinoma	105	21.9
1 bronchogenic	*99*	*20.7*
a. acinar	*62*	*12.9*
b. papillary	*37*	*7.7*
2 bronchiolo-alveolar	*6*	*1.3*
IV Large cell carcinoma	15	3.1
V Combined epidermoid and adenocarcinoma	3	0.6
IX. Carcinosarcoma	1	0.2
II.2 + III.1	1	0.2
XI Unclassified	2	0.4

This demonstrates that epidermoid carcinoma (Fig 1) was found in 48 per cent. Small cell anaplastic carcinoma (Figs. 2–4) occurred in 25 per cent. About 2/3 was of the oat cell type and 1/3 of the polygonal cell type. 22 per cent of the tumours were adenocarcinomas (Figs. 5 and 6) and among them the acinar type predominated. Bronchiolo-alveolar type (Fig 7) occurred in only 1 per cent, large cell carcinoma (Fig. 8) in 3 per cent and carcinoma of combined type (Fig 9) in 0 6 per cent. In 2 cases it was not possible to classify the tumour The degree of differentiation in epidermoid carcinoma and adenocarcinoma is shown in Table 3

Histological Tumour Type in the Biopsy Group

The result of the histological typing in the biopsy group is shown in Table 4 Epidermoid carcinoma and small cell anaplastic carcinoma occurred with approximately the same frequency in the biopsy material as in the material as a whole The frequency of adenocarcinoma though was only half that of the total material In 44 of the 289 cases (15 per cent) biopsy did not permit assessment of the tumour type

Reliability of Biopsy Typing

The reliability of tumour classification in biopsies is shown in Tables 5 and 6. In 25 (17 per cent) of the operated patients biopsy did not permit typing The pre-operative classification was correct in 89 per cent. Of the 78 cases which pre-operatively were classified as epidermoid carcinoma, 67 (86 per cent) corresponded with the final diagnosis. In 23 (92 per cent) of the 25 cases pre-operatively diagnosed as small cell anaplastic carcinoma the final diagnosis was the same In the 16 adenocarcinoma cases the pre-operative and the final classifications agreed completely

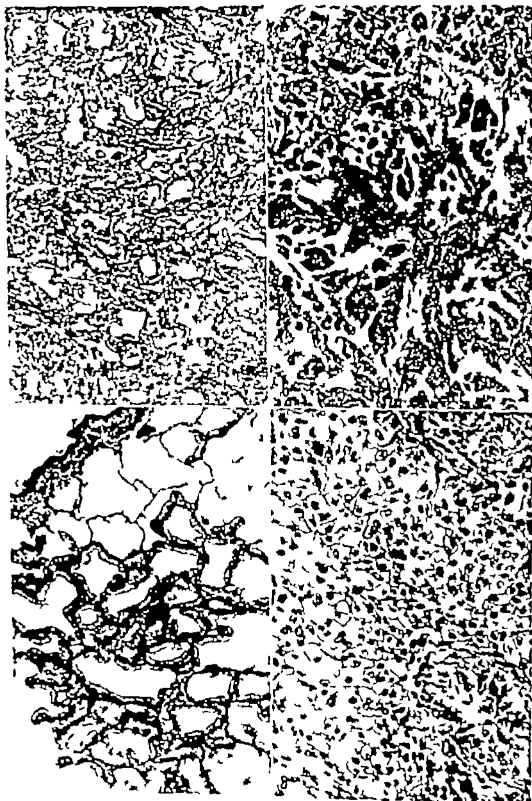
In the group non-operated patients, biopsy did not permit typing in 14 cases (14 per cent) (Table 6) The tumour classification

Fig 5 Bronchogenic adenocarcinoma, acinar type Hematoxylin-eosin

Fig 6 Bronchogenic adenocarcinoma, papillary type Hematoxylin-eosin

Fig 7 Bronchiolo-alveolar carcinoma. Hematoxylin-eosin.

Fig 8 Large cell carcinoma. Hematoxylin-v.Gieson.



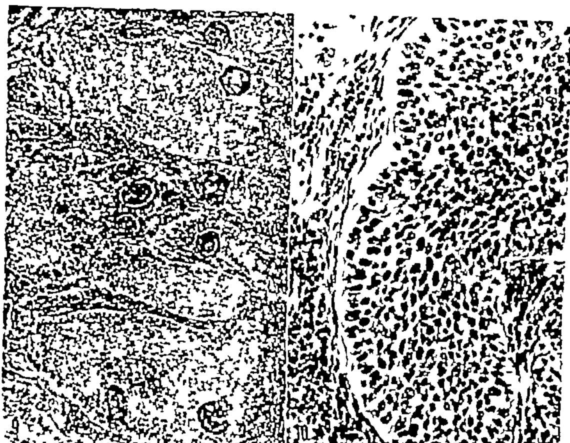


Fig. 9 Combined epidermoid and adenocarcinoma. PAS.
Fig. 10 Transitional cell carcinoma. Hematoxylin-eosin.

made at biopsy was correct in 86 per cent. Of the 34 cases of epidermoid carcinoma, 24 (71 per cent) were diagnosed correctly by biopsy. The biopsy typing was correct in all the 37 cases of small cell anaplastic carcinoma and in 11 of the 12 adenocarcinoma cases (92 per cent).

DISCUSSION

In the histological classification of our material we have rigorously applied the criteria set up by WHO. Furthermore we have subsequently adhered to the principle that if anywhere in a tumour a specific differentia

TABLE 3. *Degree of Differentiation in 231 Cases of Epidermoid Carcinoma and 105 Cases of Adenocarcinoma*

Histological type	Degree of differentiation		
	high	low	not stated
Epidermoid carcinoma	70 (30.3 %)	159 (68.8 %)	2 (0.9 %)
Adenocarcinoma			
Acinar	8	54	
Papillary	11	26	
Bronchiolo-alveolar	6		

TABLE 4. *Tumour Type Distribution in the Biopsy Group*

Histological tumour type	No.	%
Epidermoid carcinoma	136	47 1
Small cell anaplastic carcinoma	70	24.2
Adenocarcinoma	32	11 1
Large cell carcinoma	7	2.4
Unclassified	44	15.2
	289	100.0

tion was found, this finding should determine the type. Undifferentiated areas were not used for type designation, except when every area examined was undifferentiated If two specific differentiations were found the tumour was classified as combined. However we have, as other authors (*Walter* & *Price* 1955 *Azzopardi* 1959 *Whitwell* 1961 *Kreyberg* 1962 *Shinton* 1963) interpreted the existence of small groups of squamous-like

TABLE 5 *Comparison between the Results of Biopsy Typing and Final Typing of Primary Lung Cancer in 146 Patients Subjected to Excisional Surgery Percentages in Brackets Are Based on Row Totals*

Histological tumour type	At biopsy No	At biopsy %	At final typing: Epidermoid carcinoma	Small cell carcinoma	Adeno-carcinoma	Large cell carcinoma	Others
Epidermoid carcinoma	78	53.4	67 (85.9)	4	3	4	0
Small cell carcinoma	25	17 1	2	23 (92.0)	0	0	0
Adeno-carcinoma	16	11.0	0	0	16 (100)	0	0
Large cell carcinoma	2	1 4	1	0	1	0	0
Unclassified	25	17 1	16	4	4	0	1
	146	100.0	86 (58.9)	31 (21.2)	24 (16 4)	4 (2.7)	1 (0 7)

TABLE 6. *Comparison between the Results of Biopsy Typing and Final Typing of Primary Lung Cancer in 100 Patients not Subjected to Surgery Percentages in Brackets Are Based on Row Totals*

Histological tumour type	At biopsy N	At biopsy %	At final typing: Epidermoid carcinoma	Small cell carcinoma	Adeno-carcinoma	Large cell carcinoma	Others
Epidermoid carcinoma	34	34.0	24 (70.6)	4	4	2	0
Small cell carcinoma	37	37.0	0	37 (100)	0	0	0
Adeno-carcinoma	12	12.0	0	0	11 (91 7)	0	1
Large cell carcinoma	3	3.0	0	0	2	1	0
Unclassified	14	14.0	2	2	7	2	1
	100	100 0	26 (26 0)	43 (43.0)	24 (24.0)	5 (5.0)	2 (2.0)

cells with foci of keratinization, and gland like rosette forming structures in tumours of small cell anaplastic type to be characteristic of this cancer type.

As *Kreyberg* (1962) stated, in order to be both rational and useful, a classification must be *comprehensive* so that only a minimum of tumours remain unclassified. Further the criteria should be sufficiently precise and objective to permit any pathologist to type a given tumour in the same way that is, the typing should be *reproducible*

The WHO classification evidently forfills to the maximum requirements in comprehensiveness. In our material all but two of the 479 tumours could be classified. The high rate of comprehensiveness is almost certainly due to the fact that in the majority of cases a great number of slides were available for examination and that in uncertain cases special stains were used.

The extent to which a classification can be considered reproducible is naturally not only dependent on the stringency and objectivity of the criteria but also on the pathologist's ambition and ability to apply them. By comparing the frequency of different tumour types in material of similar derivation one acquires an impression of the reproducibility of a classification Another method to check the reproducibility is to allow a number of experienced pathologists to classify the same material blindly and independently of one another applying identical criteria. This method has been practised by *Yesner et al* (1965) and *Salzer* (1967) The result of these two investigations is indeed not encouraging and appears to support the opinion of many pathologists that histological classification of lung cancer is meaningless. In our opinion there is no justification for such a negative attitude In the case of *Salzer's* investigation the low rate of reproducibility would seem to a large extent to be due to the fact that the diagnostic type criteria were greatly summarized. In *Yesner et al*'s investigation the low rate of reproducibility is less easily explained.

As regards our own investigation, an impression of the reproducibility of the classification is obtained by comparing the biopsy tumour type with the final tumour type in the 146 patients subjected to surgery and the 100 patients not operated upon. In each of these groups there is correspondence of typing in approximately 90 per cent of the cases. As shown in Tables 5 and 6, the type agreement in both groups is not as complete in epidermoid carcinoma (86 and 71 per cent, respectively) as in small cell anaplastic cancer (92 and 100 per cent, respectively) and in adenocarcinoma (100 and 92 per cent, respectively)

The WHO classification has received ample praise from many sources (*Berndt* 1967 *Harrison* 1967 Annotations, The Lancet 1967 vol 1 p. 716 and Book Reviews, Canad. Med. Ass. J 1967 vol. 97 p. 1306, *Hallgrimsson* 1973) A classification is seldom so perfect, however that it can not be improved, which is also true of the WHO classification of lung tumours. In our opinion the greatest deficiency in the WHO classification is that it does not more closely relate to the type of lung cancer that *Brandt* as early as 1926 described as "gemischtzellige Uebergangskrebse mit Neigung zu Cylinder oder Stachelzellbildung" This type of cancer grows in a manner resembling epidermoid carcinoma, forming relatively wide bands. Furthermore, it is characterized by stratification, whorl formation and a basal layer of cylindrical cells arranged in a palisade-like structure (Fig. 10) However this cancer type often does not fill the criteria for epidermoid carcinoma as formulated by WHO ("tumours with keratinization of intercellular bridges") and is therefore placed in the group of small cell anaplastic cancer The tumour is indeed small celled but it differs from the small cell anaplastic type in other respects, and in our opinion it should be classified as epidermoid carcinoma of low differentiation. Obviously the board responsible for the WHO classification was not unanimous as regards to how the "transitional cell carcinoma" should be classified as in Fig 10 in the WHO publication Histological typing of lung tumours such

a tumour is recorded as "epidermoid carcinoma or small cell anaplastic carcinoma, fusiform type?"

In 1968 a meeting dealing with the histological classification of lung cancer was held in Oslo under the chairmanship of Professor Kreyberg. We then had the opportunity to forward the above viewpoint on the classification of the transitional cell carcinoma. In 1971 *Kreyberg* took up this problem for discussion writing "Epidermoid carcinomas (WHO Code No. 1) are defined as 'tumours with keratinization or intercellular bridges'. This is a precise, but also a very narrow delimitation. Stratification and whorl formation are not mentioned, whereby tumours with a 'transitional' cell epithelium are excluded. Many will find this regrettable, and if the designation 'squamous cell' carcinoma of the old tradition had been used, a field of controversy would have been removed"

Despite our critical attitude toward the delimitation of epidermoid carcinoma in the WHO classification of lung cancer we do find it to be of great value. The criteria are stringent and objective, most types are well defined, and only slight modifications are desirable. The reliability of typing is high when the criteria are consistently applied. The WHO classification will permit the clinical features and prognosis of cases in one centre to be compared with those of another

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BRIEF REPORT

G-BAND ANALYSES OF A HUMAN INTESTINAL LEIOMYOSARCOMA

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Mark, J G-band analyses of a human intestinal leiomyosarcoma. Acta path. microbiol. scand. Sect. A, *84* 538–540 1976.

The chromosomes of a human intestinal leiomyosarcoma were studied by a direct method. The tumour had a hypodiploid stemline (S = 4?) with both numerical and structural changes. These could be clarified using a G-banding method. The evolutionary pattern in the sarcoma was reminiscent of that found earlier in human meningiomas.

Key words Leiomyosarcoma, human intestinal chromosomes G-banding

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The present report deals with the cytogenetic findings by G-banding technique in a primary human leiomyosarcoma of intestinal origin. So far no such neoplasms have been studied by means of the new chromosomal methods Furthermore, if leukaemias-lymphomas are excluded, less than ten primary solid, malignant tumours in man have hitherto been completely analysed with banding techniques (references in *Mark* 1976)

Material and Methods

A 77-year-old female was subjected to ileo-caecal resection due to an obstructing small-bowel tumour with incipient perforation. The tumour was located in the wall of ileum about 10 cm proximal to valvula Bauhini. The rounded, solid neoplasm had a diameter of approximately 10 cm. The tumour was a well-differentiated leiomyosarcoma. The same histological picture was seen in an umbilical metastasis removed 8 months later At that time laparatomy revealed disseminated metastases to the abdominal cavity The general condition of the patient deteriorated during the following 16 months but she was still alive about 2 years after diagnosis.

Fresh pieces of the intestinal tumour were used for the chromosome preparation which was performed by a direct method It was described in detail earlier as was also the technique used for the G-banding (*Mark* 1975) The chromosomes were counted in 75 cells. The karyotype analyses were made by photography and they comprised 53 cells. The nomenclature follows that of the Paris Conference (1971) with two exceptions (1) S is used as a symbol for the stemline; (2) all structurally changed chromosomes are termed markers

Results

The chromosome counts are shown in Table 1 There was a definite mode at S = 4 a restricted spread around the modal number and only a few polyploid cells. The results of the karyotype analyses are summarized in Table 2. The stemline cells (marked with S in Table 2 Figs. 1 and 2) as most variant cells, contained two markers, namely one No. 1 with short-arm deletion (break point p12 13) and one No 11 with long-arm deletion (break point q13-14) The deleted segments were not translocated onto any other chromosome(s) No further marker types were seen in any of the karyotyped cells

The banding analyses revealed 11 different karyotypes in addition to that of the S-cells (cf Table 2) It seems beyond doubt, however that the one with a normal complement represents a non-neoplastic stromal or inflammatory cell. The structural and/or numerical deviations characterizing the other

TABLE 1 *Chromosome Counts*

Chromosome numbers										Total cells
38	39	40	41	42	43	44	45	46	84	
1	1	4	12	44	7	3	–	1	2	75

TABLE 2. *Karyotypic Observations*

Karyotype no.	Number of cells	Chromosome number	Karyotypic deviations 1	7	9	11	13	14	15	16	19	22
1	1	46	—	—	—	—	—	—	—	—	—	—
2	2	44	—	—	—	—	—	—	—1	—	–	—1
3	1	43	—	+1	—	—	–	—1	—1	—1	—	—1
4	2	43	p—	+1	—	—	—	—1	—1	—1	—	—1
5	17	4 = 8	p—	+1	—	q—	—1	—1	—1	—1	—	—1
6	1	42	p—	+1	—	—	—1	—1	—1	—1	—	—1
7	1	42	p—	+1	—1	—	—1	—1	—1	—1	+1	—1
8	1	42	p—	+1	—1	q—	—1	—1	—1	—1	+1	—1
9	2	42	p— +1	+1	—1	q— —1	—1	—1	—1	—1	+1	—1
10	3	41	p—	+1	—	—1	—1	—1	—1	—1	—	—1
11	1	41	p—	—	—	q— —1	—1	—1	—1	—1	+1	—1
12	1	40	p—	+1	—1	—1	—1	—1	—1	—1	—	—1



Fig 1 Karyotype of stemline cell, S = 42 b, and d partial karyotypes of three S-cells showing the normal and the deleted Nos. 1 and 11 respectively × 2600



Fig. 2 Metaphase plate of a stemline cell (S = 4) the arrows point to the two marker chromosomes (cf. Fig. 1)

10 karyotypes demonstrate their usually close relation to the S-cells. It was also possible to analyse one of the two 84-chromosome cells almost completely its karyotype agreed with that of a doubled S-cell.

Discussion

The results of the present study merit notice with regard to the following facts and conclusions: (1) the stemline cells and a great number of variant cells in the leiomyosarcoma could be completely analysed with regard to their banding pattern (2) the karyotypic findings showed very clearly the monoclonal origin of the tumour cell population (3) the results of the banding analyses suggested a step-by-step development of the clonal evolution. As to the last-mentioned point, the findings indicated that the development had originated with loss of one No. 13 and one No. 22, continued with loss of one No. 14 and one No. 18, gain of one No. 7 and deletion of one No. 1 and then led to the establishment of the stemline by loss of one No. 13 and deletion of one No. 11. The additional changes observed in some hypomodal variant cells might foreshadow characteristics of subsequent steps in the evolutionary chain. This theoretical interpretation of the karyotypic data agrees well with the principal features of the chromosomal progression in another thoroughly studied, human tumour type namely the meningioma (*Mark* 19 6) In this context it is worth noting that the leiomyosarcoma shows several evolutionary features typical of meningiomas i.e. a hypodiploid progression, early involvement of No. 22, and later, superimposed, deviations affecting No. 1 and the *D* group chromosomes. Further studies of leiomyosarcomas (and also other mesodermal tumours) are necessary to clarify whether or not these similarities are coincidental.

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